THE ANTICOAGULANT AND ANTITHROMBOTIC PROPERTIES OF HUMAN PROTHROMBIN FRAGMENT 1.2

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ABSTRACT

A method for the preparation of human prothrombin fragment 1.2 (F1.2) from freshly clotted plasma was developed. Addition of exogenous F1.2 to citrated normal human plasma prolonged prothrombin time and activated partial thromboplastin time by 0.9 and 2.4 s/μM F1.2, respectively. Delayed thrombin generation was not attributed to inhibition of tissue factor activity nor inhibition of factor X activation but to interference with phospholipid interactions in the prothrombinase complex. In mice, 500 μg (14 μM) of F1.2 gave 100% protection from tissue factor lethality, whereas 6 units (3 μM) of heparin was required for 100% protection. Either dose of F1.2 or heparin protected mice from thrombin-induced death. However, 500 μg of F1.2 was not effective in protecting mice from lethal effect of factor Xa and cephalin, while 0.75 units of heparin prevented such lethality. These findings demonstrate that human prothrombin F1.2 has anticoagulant and antithrombotic properties.

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DIP-F	diisopropyl phosphofluoridate
EDTA	ethylenediaminetetraacetic acid
F1.2	fragment 1.2
h	hour
HAT	100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine.
L	liter
min	minute
s	second
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminoethane

Tris-buffered saline	0.15 M NaCl, and 50 mM Tris-HCl to pH 7.4.

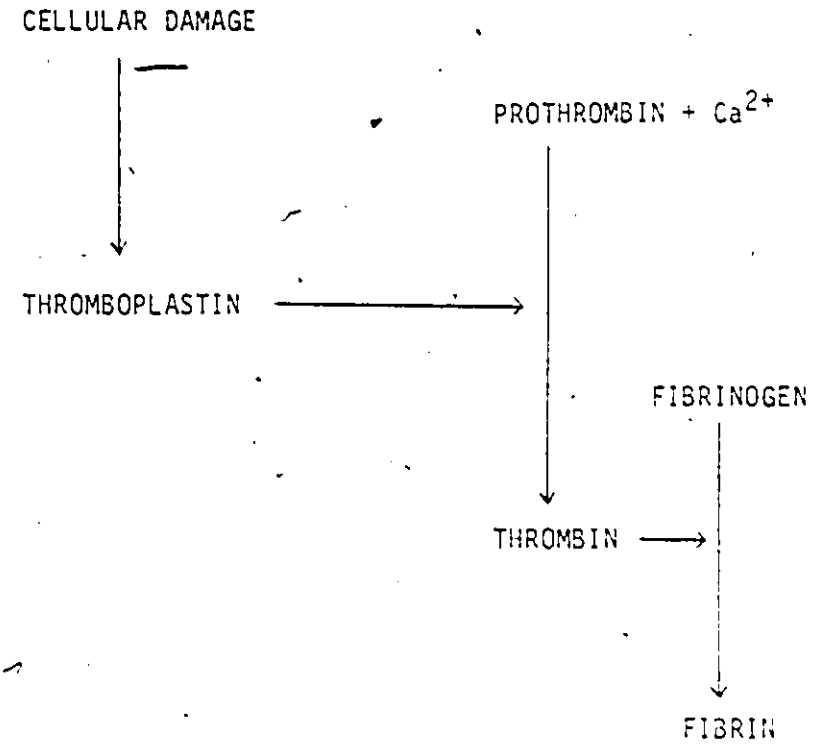
CHAPTER 1

INTRODUCTION

1.1. EVOLUTION OF THE COAGULATION SCHEME

For more than a century, contact between damaged tissue and blood has been known to initiate the clotting process. The contribution of tissue factor to this phenomenon was first demonstrated by de-Blainville in 1834, when he injected tissue extract intravenously into animals and observed immediate death of the animals from intravascular thrombi. Following this, the existence of separate inactive entities in blood which could be triggered into sequential reactions by calcium ions and an activator released from cellular damage became evident from the work of many researchers (reviewed by Biggs & MacFarlane, 1962; Biggs, 1972). Subsequently, Morawitz (1905) proposed his classical 'four factor' theory of blood coagulation (Fig. 1). This hypothesis recognized four essential components of blood coagulation : fibrinogen (factor I), prothrombin (factor II), tissue factor or thromboplastin (factor III), and calcium ions (factor IV). Morawitz postulated that in normal circulation

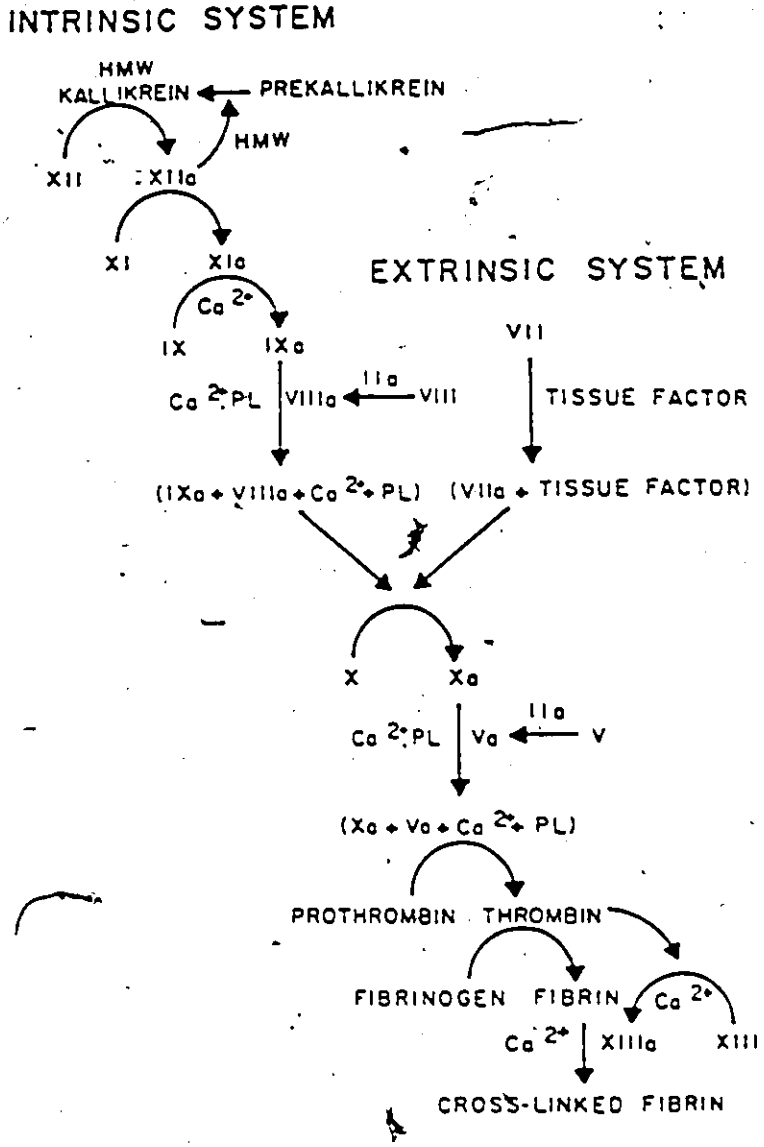
Fig. 1 The 'four factor' theory of blood coagulation from Biggs (1972).


CELLULAR DAMAGE
PROTHROMBIN + Ca^{2+}
THROMBOPLASTIN
FIBRINOGEN
THROMBIN
FIBRIN

blood remained fluid because prothrombin and fibrinogen were inherently stable. Following tissue damage, thromboplastin entered the blood and reacted with prothrombin, which in turn reacted with fibrinogen to form the insoluble strands of fibrin. As we now know, this theory presents in essence only the penultimate stage of the coagulation reactions. The complexity of the blood coagulation system with its nearly 20 different components was not recognized until more than fifty years after Morawitz's initial proposal.

The cascade or waterfall model for blood coagulation, proposed by Macfarlane (1964) and Davie and Ratnoff (1964), is based on numerous experimental observations and in particular the discovery of coagulation factors V to XII. They proposed that blood coagulation proceeds via a biochemical amplification system in which, sequentially, one serine proteinase activates a succeeding proenzyme to a proteinase until prothrombin is converted to thrombin (Fig. 2). In this model, 2 routes for the initiation of blood coagulation were proposed: the intrinsic (contact) pathway initiated with factor XII in contact with artificial surfaces, and the extrinsic pathway initiated with factor VII and tissue factor. The "intrinsic" pathway is so named because it operates strictly with components normally found in blood which upon exposure to glass

Fig. 2 An overview of coagulation pathways from Bang (1984).


INTRINSIC SYSTEM
HMW KALLIKREIN
PREKALLIKREIN
HMW
XII
XIIa
XI
XIa
Ca 2+
IX
IXa
EXTRINSIC SYSTEM
VII
Ca 2+ PL
VIIIa
IIa
VIII
TISSUE FACTOR
(IXa + VIIIa + Ca 2+ PL)
(VIIa + TISSUE FACTOR)
X
Xa
Ca 2+ PL
Va
IIa
V
(Xa + Va + Ca 2+ PL)
PROTHROMBIN
THROMBIN
FIBRINOGEN
FIBRIN
Ca 2+
Ca 2+
XIIIa
XIII
CROSS-LINKED FIBRIN

or other negatively charged surfaces initiates coagulation. The term "extrinsic" was applied because tissue extract required to trigger coagulation was extrinsic to blood. While the initiation and propagation reactions differ, the 2 pathways converge at the activation of factor X. In both cases, activation of factor X involves proteolytic cleavage of a single Arg_{51}-Ile_{52} bond of the heavy chain of factor X and the liberation of an activation glycopeptide (Fujikawa et al., 1974; Jesty and Nemerson, 1974). The factor Xa generated by either pathway converts prothrombin to thrombin (discussed in next section). Subsequent limited proteolysis of fibrinogen by thrombin results in the formation of fibrin monomers, which in turn will polymerize spontaneously, and on cross-linking by factor XIIIa result in the formation of insoluble fibrin network.

Due to early recognition of hemorrhagic diathesis associated with factor VIII and IX deficiencies, the importance of the intrinsic pathway is well documented (Levine, 1987). More recently, it has been demonstrated that factor VII, tissue factor, and Ca^{2+} in addition to being an activator of factor X can also serve as a very potent activator of factor IX (Osterud and Rapaport, 1977). Further, the clinical significance of factor VII is evident from reports of bleeding and thrombotic

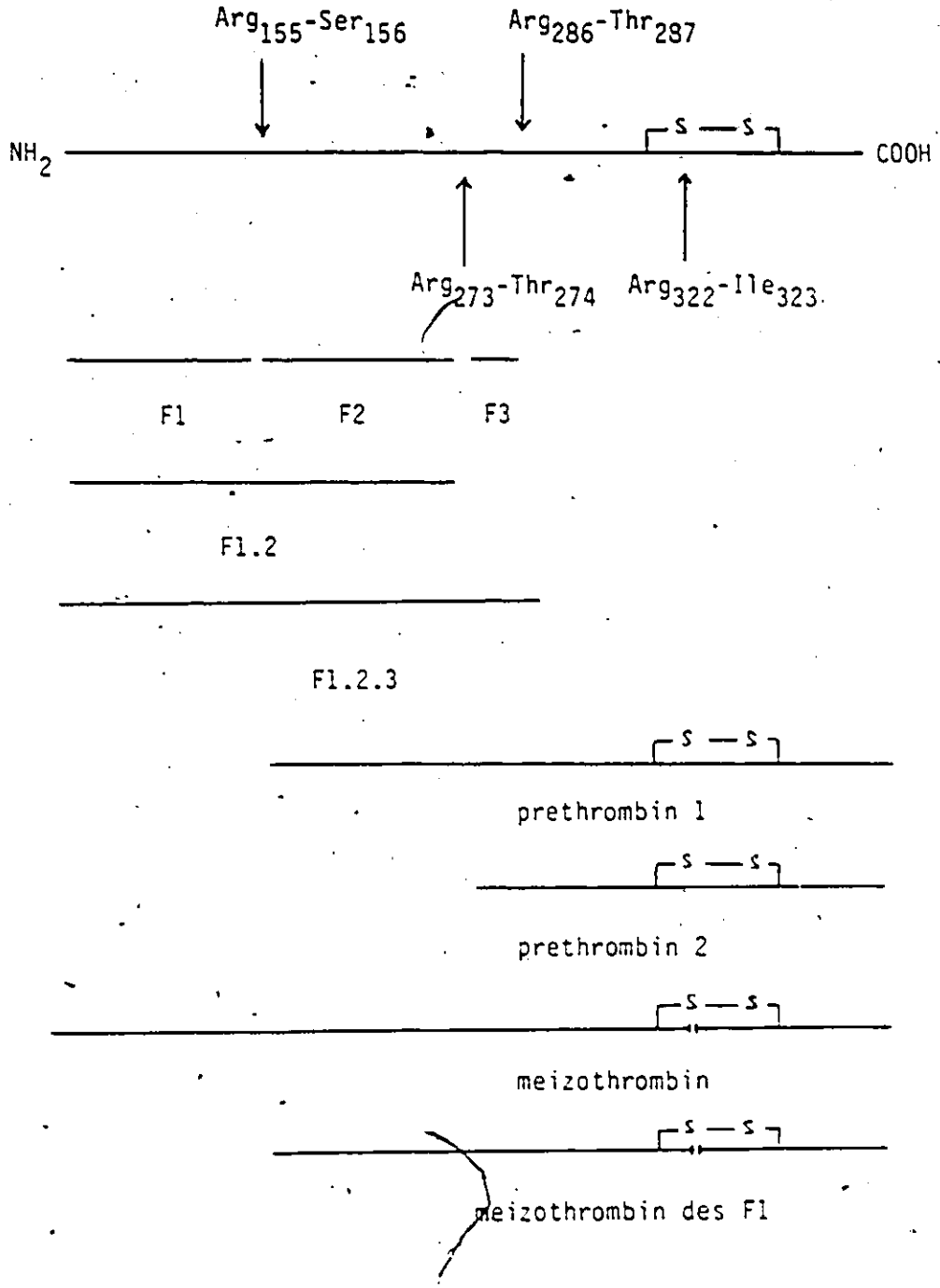
tendencies in patients with factor VII deficiencies (Gershwin and Gude, 1973; Triplett et al., 1985). Thus, the role of extrinsic pathway in physiologic coagulation cannot be overlooked.

1.2. ACTIVATION OF PROTHROMBIN

Prothrombin is a single chain glycoprotein with a molecular weight of approximately 72,500 daltons (Magnusson et al., 1975; Suttie and Jackson, 1977). The primary structures of human and bovine prothrombins have been elucidated, and there are 581 and 582 amino acid residues in the human and bovine molecule, respectively (Mann et al., 1981). Prothrombin occurs in human plasma at a concentration of about 0.15 g/L (2 μM) with a range of 110 to 212 mg/L (McDuffie et al., 1979).

Prothrombin is susceptible to proteolysis by factor Xa and thrombin (Suttie and Jackson, 1977; Jackson, 1987). The bonds hydrolyzed are illustrated in Fig. 3. Factor Xa cleaves human prothrombin at two sites Arg_{273}-Thr_{274} and Arg_{322}-Ile_{323} (Downing et al., 1975; Rosenberg et al., 1975a). Since bovine prothrombin contains a single residue insertion at position 4 in the sequence, Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324} are the corresponding bonds in bovine prothrombin cleaved by factor Xa

Fig. 3 A diagrammatic representation of human prothrombin showing peptide bonds cleaved by factor Xa and thrombin. Factor Xa cleavage sites are indicated by arrows below the protein and thrombin cleavage sites are indicated by arrows above the protein.


Arg155-Ser156
Arg286-Thr287
S — S
NH2
COOH
Arg273-Thr274
Arg322-Ile323
F1
F2
F3
F1.2
F1.2.3
S — S
prethrombin 1
S — S
prethrombin 2
S — S
meizothrombin
S — S
meizothrombin des F1

(Esmon and Jackson, 1974a; Owen et al., 1974). The peptide bonds in human prothrombin susceptible to the action of thrombin are Arg_{155}-Ser_{156} and Arg_{286}-Thr_{287}, whereas in bovine prothrombin only the Arg_{156}-Ser_{157} bond is cleaved by thrombin (Downing et al., 1975; Esmon et al., 1974a). In addition, the Arg_{155}-Ser_{156} and Arg_{286}-Thr_{287} bonds in human prothrombin have been reported to be susceptible to hydrolysis by either thrombin or factor Xa (Downing et al., 1975; Rosenberg et al., 1975a; Lau et al., 1979).

The nomenclature for the various proteolytically derived intermediates or products of prothrombin is designated by whether or not they contain the amino acid sequence which ultimately becomes thrombin, i.e., prethrombins contain the sequence of thrombin and prothrombin fragments do not (Jackson, 1977). Thus, the recommended terminology for human prothrombin derivatives is as follows: fragment 1 (residues 1-155), fragment 2 (residues 156-273), fragment 3 (residues 274-286), fragment 1.2 (residues 1-273), fragment 1.2.3 (residues 1-286), prethrombin 1 (residues 156-581), prethrombin 2 (residues 274-581), meizothrombin (two chain structure comprised of residues 1-322 and 323-581 covalently linked by disulfide bonds), meizothrombin des fragment 1 (two chain structure comprised of residues 156-322 and 323-581 covalently linked by disulfide

bonds).

Studies on factor Xa-catalyzed conversion of prothrombin into thrombin indicate that the reaction proceeds primarily via two pathways. The major difference between the two pathways resides in the order of bond cleavage of prothrombin by factor Xa (Jackson, 1987). It appears that in the absence of the prothrombin activation accessory component, factor Va, the first peptide bond hydrolyzed is Arg_{274}-Thr_{275} and prethrombin 2 occurs as the intermediate in the formation of thrombin (Esmon et al., 1974b; Krishnaswamy et al., 1986). However, meizothrombin is the intermediate involved in prothrombin activation catalyzed by prothrombinase complex (factor Xa, factor Va, phospholipids and Ca^{2+}) as Arg_{323}-Ile_{324} is the first peptide bond hydrolyzed (Krishnaswamy et al., 1986; Rosing et al., 1986). In either case, activation of prothrombin leads to formation of fragment 1.2 and thrombin although the order of bond cleavage may be reversed (Jackson, 1987).

In the activation of prothrombin, the isolation of fragment 1, fragment 2, fragment 1.2, and fragment 1.2.3 have all been reported (Aronson et al., 1977; Aronson et al., 1980; Govers-Riemslag et al., 1985; Ofosu et al., 1986; Ofosu et al., 1987; Rabiet et al. 1986). The precise reaction conditions

that favor the formation of these fragments are not well defined. It is possible that F1.2.3 is a transient intermediate and that F1 and F2 are degradation products. Further, Aronson et al. (1977) have shown that F1.2 persists in freshly clotted human blood for hours in a plasma milieu; and purified F1.2, however, is easily degraded by thrombin into F1 and F2.

Functionally, prothrombin F1 with its 10 gamma-carboxyglutamic acid (Gla) residues is the vitamin K-dependent portion of prothrombin as vitamin K is required for the post-ribosomal carboxylation of specific glutamic acid residues (Esmon et al., 1975; Jackson and Suttie, 1977). The Gla residues confer on the molecule the ability to bind Ca^{2+}, which mediate the binding of prothrombin to coagulant phospholipids through Ca^{2+} "bridges" (Gitel et al., 1973; Dombrose et al., 1979). The function of F2 appears to be related to interactions with factor Va (Esmon and Jackson, 1974b; Guinto and Esmon, 1984). The carboxyl-terminal half represents the enzymic functional domain of prothrombin, and expression of active serine site of enzyme requires the hydrolysis of Arg_{322}-Ile_{323} (Jackson, 1987).

The efficient conversion of prothrombin to thrombin requires the assembly of the prothrombinase complex (Suttie and

Jackson, 1977; Jackson, 1987). The components which constitute the complex are factor Xa, Ca^{2+}, phospholipids and factor Va. Factor Xa is the catalytic component of the complex and the other components serve to accelerate the rate of the factor Xa-catalyzed reactions.

Factor Xa, a serine protease, hydrolyzes 2 peptide bonds in prothrombin: the arginyl-threonyl bond between the pro-half (prothrombin fragment 1.2) and the thrombin-forming (prethrombin 2) half of the molecule, and the arginyl-isoleucyl peptide bond between the 2 chains of thrombin (discussed in previous paragraphs). The order of bond cleavage in prothrombin by factor Xa may vary pending on the reaction condition (Jackson, 1987; Krishnaswamy et al., 1986). However, factor Xa in the absence of accessory components can still catalyze the conversion of prothrombin to thrombin albeit at a rate insufficient to support blood coagulation (Jackson, 1987).

In prothrombin activation, Ca^{2+} alter the rate of cleavage by both factor Xa and thrombin as well as mediate the binding of prothrombin and factor Xa to the phospholipid bilayer membrane surface (Papahadjopoulos et al., 1964; Esnouf and Jobin, 1965; Barton and Hanahan, 1969; Jackson et al., 1974; Nelsestuen and Broderius, 1977; Nelsestuen and Lim, 1977; Dombrose et al., 1979; Mayer et al., 1983); Ca^{2+} can also

affect factor Va subunit interactions (Esmon, 1979; Guinto and Esmon, 1982).

The role of phospholipids in the factor Xa catalyzed activation of prothrombin is to provide a negatively charged polar surface for the assembly of coagulation factors. The presence of phospholipids in the reaction mixture causes a decrease in Michaelis-Menten constant (K_m) of the enzymic action of factor Xa on prothrombin (Rosing et al., 1980; Jackson, 1987). Consequently, the effect of phospholipids is to decrease the enzyme saturation requirement to that occurring in plasma or conversely to increase the substrate concentration at the lipid surface.

The presence of factor Va in the prothrombin activation reaction mixture leads to an increase in maximal velocity (V_{max}), i.e., factor Va can accelerate the enzymic action of factor Xa on prothrombin, presumably by bringing the substrate prothrombin towards the active site of the enzyme factor Xa (Nesheim et al., 1979; Rosing et al., 1980; Nesheim et al., 1980). In addition, factors Va and Xa can form 1:1 stoichiometric calcium-dependent complexes, and the formation of the complex is stimulated by acidic phospholipids (Nesheim et al., 1979; Lindhout et al., 1982). However, factor Va can associate with membranes directly by interacting with the polar

head groups of the acidic phospholipids (Kandall et al., 1975; van de Waart et al., 1983).

The enhancement effects of the various components of the prothrombinase complex on prothrombin activation have been summarized by Jackson (1987). A comparison of the relative rate of thrombin formation shows that the addition of phospholipids to prothrombin, factor Xa and Ca^{2+} can increase thrombin formation by 50-fold; whereas, the addition of factor Va to the reaction mixture in the absence of phospholipids can increase the rate of thrombin formation by 350-fold. The combined effect of phospholipids and factor Va accelerate thrombin formation by 20,000-fold, a value that approximates the product of the individual enhancement effects of phospholipids and factor Va. With factor Xa, platelets and Ca^{2+}, the enhancement effect on thrombin generation is 300,000-fold. Thus, the assembly of the prothrombinase complex brings about efficient prothrombin activation. The interrelationship of the components of the prothrombinase complex with prothrombin is shown in Fig. 4 (Jackson, 1987).

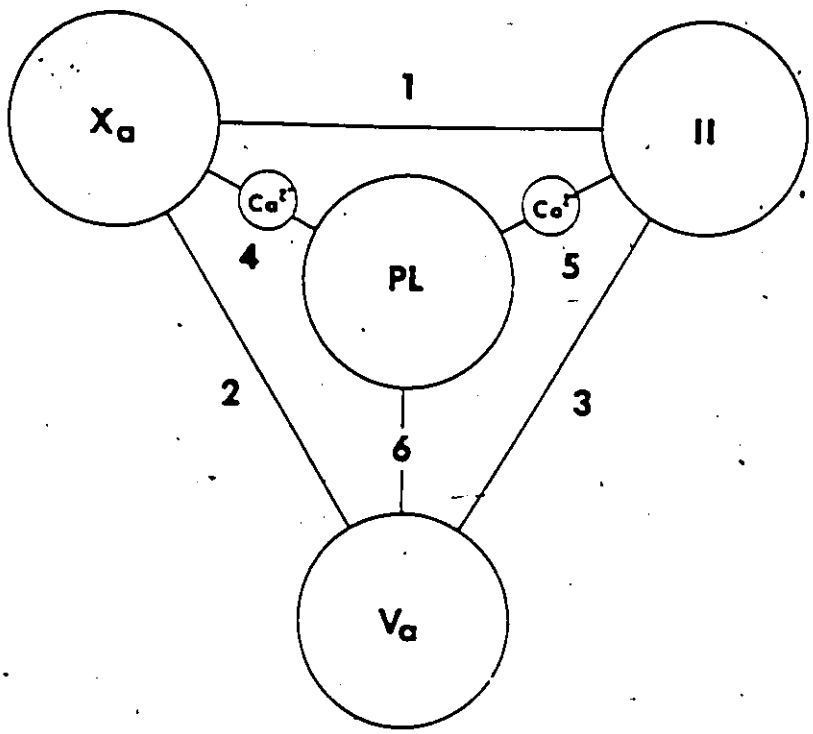
1.3. NATURAL INHIBITORS OF COAGULATION

For the maintenance of normal hemostasis, the series of

Fig. 4 A diagrammatic representation of interactions among the components of the prothrombin activation complex from Jackson (1987).

Interactions:

(1) Factor Xa-prothrombin
(2) Factor Xa-factor Va
(3) Prothrombin-factor Va
(4) Factor Xa-Ca^{2+}-phospholipid membrane
(5) Prothrombin-Ca^{2+}-phospholipid membrane
(6) Factor Va-phospholipid membrane.


X_a
1
II
Ca^{2+}
Ca^{2+}
4
PL
5
2
3
6
V_a

linked proteolytic reactions in the coagulation cascade is limited to the formation of an extravascular hemostatic plug at the site of injury, otherwise the development of an initial pathologic stimulus may lead to vascular thrombus formation or disseminated intravascular coagulation. Studies of natural anticoagulant mechanisms indicate that, aside from mechanisms that are inherent properties of the endothelium and other cell surfaces, plasma has a number of antiproteinases that can inactivate specific activated coagulation factors under certain conditions.

1.3.1. BIOCHEMICALLY CHARACTERIZED PROTEINS WHICH CAN ACT AS INHIBITORS OF COAGULATION

Some of the known plasma proteins that can act as inhibitors of activated coagulation factors, at least in vitro, are antithrombin III, heparin cofactor II, protein C, protein S, α_1-proteinase inhibitor, α_2-macroglobulin, and C1 esterase inhibitor of the complement system.

1.3.1.1. ANTITHROMBIN III AND HEPARIN COFACTOR II

At the turn of this century, Contejean (1895) and

others recognized that thrombin gradually lost activity when added to defibrinated plasma or serum. On this basis, it was thought that a specific inactivator of thrombin, antithrombin, must be present in plasma under normal physiologic conditions. Investigations of plasma antithrombin activities led to the widely adopted classification of mechanisms responsible for thrombin inhibition proposed by Seegers et al. (1954). Antithrombin I was defined as adsorption of thrombin on fibrin; antithrombin II designated the plasma cofactor required for heparin's anticoagulant action; antithrombin III was employed to describe the entity, present in plasma and serum, which neutralizes thrombin in a progressive fashion; and antithrombin IV referred to the antithrombin effects of diethyl ether-treated plasma. Although the term antithrombin IV was proposed, it has never been demonstrated to be different from antithrombin III (Seegers et al., 1954). In the following two years, the work of Monkhouse et al. (1955) and Waugh et al. (1956) indicated that plasma antithrombin activity (antithrombin III) and plasma heparin cofactor (antithrombin II) are intimately related, and that heparin acts to accelerate, by 50- to 100-fold, the rate at which antithrombin neutralizes thrombin in plasma. The isolation of the antithrombin protein first by Abildgaard (1968), then Rosenberg

and Damus (1972) confirmed the relationship between antithrombin III and heparin. Antithrombin III neutralizes the activity of thrombin by formation of a very stable 1:1 stoichiometric complex. The relative slow rate of complex formation is dramatically accelerated in the presence of heparin while the basic mechanism of complex formation is not altered by heparin. Antithrombin III has a wide spectrum of antiproteinase activity, and in addition to thrombin it inhibits the activated coagulation factors XIIa, XIa, IXa, and Xa (Damus et al., 1973; Gitel and Wessler, 1975; Rosenberg et al., 1975; Stead et al., 1976; Kurachi et al., 1976; Chan et al., 1977). Only factor VIIa activity is poorly neutralized by antithrombin III (Godal et al., 1974; Broze and Majerus, 1980). The first family with congenital antithrombin III deficiency was described in 1965 by Egeberg. The clinical manifestations in most affected family members were recurrent venous thromboses and pulmonary embolisms. Subsequently, numerous additional antithrombin III deficiency families with repeated thrombotic events have been reported (Rosenberg, 1987). Thus, antithrombin III plays a physiologic role in the maintenance of normal fluidity of circulating blood.

Recently, another plasma thrombin inhibitor, antigenically different from antithrombin III, was identified

and purified (Briginshaw and Shanberge, 1974; Tollefsen and Blank, 1981; Tollefsen et al., 1982). It also inhibits thrombin by forming a covalent 1:1 molar enzyme-inhibitor complex, but it does not inhibit factor Xa. The rate of inhibition of thrombin by this inhibitor is greatly enhanced in the presence of heparin and dermatan sulfate. Therefore, Tollefsen et al. referred to it as heparin cofactor II. The physiologic significance of this protein remains to be determined, although heparin cofactor II deficiency associated with thrombosis has been described in two families (Sie et al., 1985; Tran et al., 1985).

1.3.1.2. PROTEIN C AND PROTEIN S

Activated protein C was first described in 1960 as autoprothrombin II-A, whereby the designation "A" was intended to reflect its anticoagulant properties (Mammen et al., 1960). Since this anticoagulant activity developed when preparations of bovine prothrombin were incubated with thrombin, Seegers and his co-workers assumed that it was derived from prothrombin. However, a decade later, Marciniak (1970, 1972) showed that autoprothrombin II-A is not derived from prothrombin but is a separate protein. Then, in 1976, Stenflo described a

presumably hitherto unknown vitamin K-dependent protein, which he named protein C, as it was the third peak isolated in his fractionation scheme. No function was associated with protein C until Seegers et al. (1976) showed that the protein was immunologically indistinguishable from autoprothrombin II-A.

Biochemical studies of protein C demonstrated that it is a serine proteinase zymogen, which can be converted to a serine proteinase, activated protein C, by proteolytic cleavage of a peptide bond at Arg_{14}-Ile_{15} with concomitant release of a tetradecapeptide of 2,000 daltons molecular weight from the heavy chain (Stenflo, 1976; Kisiel et al., 1976; Kisiel et al., 1977). Either thrombin, the purified factor X activator from Russell's viper venom, or trypsin may catalyze this reaction.

Initially, it was thought that the role of protein C in hemostasis was not of major physiologic importance because of the slow rate of activation of protein C by thrombin observed in in vitro experiments (Esmon et al., 1976; Kisiel et al., 1977). However, this view was reversed with the identification and isolation of a cofactor, named thrombomodulin, on the surface of endothelial cells. It was observed that thrombomodulin increases the rate of thrombin-catalyzed activation of protein C by more than 20,000-fold (Esmon and Owen, 1981; Esmon et al., 1982). It is of interest to note

that the binding of thrombin to thrombomodulin brings about a change in the substrate specificity of thrombin, i.e., this binding inhibits thrombin-catalyzed fibrin formation and factor V activation, while the activation of protein C is enhanced (Esmon, 1987).

Activated protein C has anticoagulant properties and prolongs the activated partial thromboplastin time of normal plasma by inactivating the coagulation factors Va and VIIIa (Kisiel et al., 1977; Marlar et al., 1982). This inactivation occurs as a result of proteolysis of factors Va and VIIIa by activated protein C (Walker et al., 1979; Vehar and Davie, 1980; Canfield et al., 1982; Comp et al., 1982; Suzuki et al., 1983; Fulcher et al., 1983). In addition, the inactivation of factors Va and VIIIa has been shown to proceed at a much faster rate in the presence of phospholipids and $CaCl_2$. More recent studies indicate that factor Va inactivation by activated protein C can be further enhanced by another vitamin K-dependent protein, protein S named after Seattle the city where it was characterized initially (DiScipio et al., 1977; Walker, 1980). Thus, it appears that the formation of a multiprotein complex composed of activated protein C, protein S, phospholipids and Ca^{2+} results in the maximal rate of factor Va inactivation (Walker, 1980; Walker, 1981).

Recent studies have established that homozygous protein C-deficient patients develop purpura fulminans, a coagulation disease which can be corrected with concentrates rich in protein C (Esmon, 1987). In addition, studies of protein C levels in normal individuals, patients with idiopathic thromboembolic disease, and patients with other physiologic and clinical condition, as well as family studies of protein C deficiency suggest that deficiency of protein C is associated with an increased tendency towards thrombosis (Gardiner and Griffin, 1983). Thus, protein C may play a major physiologic role in the regulation of blood coagulation as part of a thrombin-dependent feedback inhibition of coagulation pathways (Esmon, 1987).

1.3.1.3. α_1-PROTEINASE INHIBITOR AND α_2-MACROGLOBULIN

Plasma proteinase inhibitors, such as α_1-proteinase inhibitor and α_2-macroglobulin have the capability of inactivating some coagulation factors *in vitro*. α_1-Proteinase inhibitor inactivates thrombin, factor Xa, and kallikrein (Beatty *et al.*, 1980; Ellis *et al.*, 1982; Schapira *et al.*, 1981). α_2-Macroglobulin also inactivates thrombin, factor Xa, and kallikrein (Downing *et al.*, 1978; Ellis *et al.*, 1982;

Harpel, 1970). The exact role of these inhibitors in vivo is not known. Patients with a deficiency of α_1-proteinase inhibitor appear to be susceptible to the development of pulmonary emphysema and hepatic cirrhosis (Laurell and Eriksson, 1963); and patients with α_2-macroglobulin deficiency have no clinical symptoms (Bergqvist and Nilsson, 1979). Deficiency of these proteinase inhibitors seems not to have an abnormal effect on the hemostatic system and apparently is not associated with thromboembolic diseases.

1.3.1.4. C1 ESTERASE INHIBITOR

C1 esterase inhibitor is named for its capacity to inhibit the first component of the complement system (Ratnoff and Lepow, 1957; Pensky et al., 1961). In addition, C1 esterase inhibitor has been shown to inhibit, in vitro, plasma kallikrein, factor XIIa and factor XIa (Ratnoff et al., 1969; Gigli et al., 1970; Forbes et al., 1970; Schreiber et al., 1973). Although C1 esterase inhibitor is recognized as a major inhibitor of the contact coagulation factors (Cooper and Cochrane, 1983; Salvesen et al., 1983; de Agostini et al., 1984), congenital deficiency of C1 esterase inhibitor causes angioneurotic edema with no abnormality in the hemostatic

system (Donaldson and Evans, 1963). This apparent paradox may be related to the observation that patients with factor XII deficiency or prekallikrein deficiency do not show signs of hemorrhagic diathesis, and patients with factor XI deficiency have a relatively mild bleeding tendency (Ratnoff and Saito, 1979). Thus, the markedly prolonged partial thromboplastin times observed from patients with contact coagulation factor deficiency remain a laboratory curiosity and the physiologic role of C1 esterase inhibitor in hemostasis is uncertain.

1.3.2. OTHER UNCHARACTERIZED COMPONENTS IN PLASMA OR SERUM WHICH CAN ACT AS INHIBITORS OF COAGULATION

The existence of a factor or factors in serum which can inhibit tissue factor initiated coagulation was demonstrated independently by Schneider (1947) and Thomas (1947). Both used an *in vivo* mouse assay system, based on the ability of intravenous injections of placental tissue extracts to kill mice. Both observed that death caused by tissue factor could be prevented by heparin or preincubation of the tissue factor with serum. Thomas observed further that the inhibitory activity appeared to be due to the combined effects of two factors in serum. The first component could be precipitated by

$(NH_4)_2SO_4$ at 25% to 50% saturation, and could be inactivated by heating at 65°C for 30 min. The second component was dialyzable, thermostable, and appeared to be calcium. These serum factors could be removed partially by absorption with tissue factor. Interestingly, there was little evidence of inhibition of the thromboplastin activity of tissue factor by serum when in vitro assay systems were used. In fact, the incubation of thromoplastin with dilute serum and calcium appeared to result in acceleration of clotting. Thus, it appears that the incubation of serum and tissue factor can promote both procoagulant and anticoagulant activities.

Since the in vivo experimental results of Schneider and Thomas, a number of studies have been conducted to isolate and characterize the inhibitor. For example, Tocantins and Carroll (1949) presented data to support the idea that an inhibitor of extrinsic pathway exists in normal plasma. They isolated a lipid inhibitor, which they called antithromoplastin, from the methanol-soluble, diethyl ether-soluble fraction of plasma. This inhibitor of clotting, which apparently acts as a thromboplastin antagonist, can also be found in Cohn Fraction IV (Tocantins and Carroll, 1949).

Using a one-stage prothrombin time assay system, Lanchantin and Ware (1953) observed that incubation of serum,

tissue factor and $CaCl_2$ resulted in initial acceleration of clotting time followed by inhibition of clotting. Further, when $BaSO_4$ adsorbed serum was incubated with tissue factor and $CaCl_2$, a marked inhibition of tissue factor activity was apparent almost immediately. The inhibitor was heat-labile and non-dialyzable, did not adsorb to $BaSO_4$ and required Ca^{2+} for its inhibitory activity. This inhibitor was also present in plasma except the inhibitory effect was somewhat masked by procoagulant activities (Lanchantin and Ware, 1953).

The results of others regarding the presence of an inhibitor in serum was investigated by Hjort in 1957. Upon studying the inhibitory effects of both Ba^{2+} adsorbed and unadsorbed sera, Hjort concluded that there was a heat-labile, non-dialyzable, Ca^{2+}-dependent factor present in $BaSO_4$-adsorbed serum, which could inactivate factor VIIa, but not tissue factor and factor VII. In addition, he observed that unadsorbed serum could inactivate both factor VIIa and tissue factor. The rates of inactivation of factor VIIa and tissue factor by unadsorbed serum were the same. However, adsorbed serum could inactivate factor VIIa at a faster rate than unadsorbed serum. The reason for the difference between the inactivation rates in adsorbed and unadsorbed sera was not explained. It is impossible to determine, from the results of

Hjort's study, whether barium sulfate absorption was removing procoagulants only or a combination of procoagulants and anticoagulants.

From the brief literature review presented in the preceding paragraphs, it is apparent that there is good evidence to support the notion that there is/are factor(s), present in plasma or serum, which can inhibit tissue factor initiated coagulation. However, it is difficult to conceive the nature and mode of action of the inhibitory substances from the results of work performed between 1949 and 1957. The major reason for this difficulty is due to the complexity of the coagulation cascade; inhibition studies cannot be performed effectively without purified materials. Hence, investigations on this topic practically halted for the next 25 years as most researchers in the field of thrombosis and hemostasis were taken up with purification and characterization of coagulation factors. With most coagulation factors purified to homogeneity, researchers are now better able to reinvestigate the subject of inhibitors of coagulation.

The concept that a lipid component could exert an inhibitory effect on coagulation was reexamined with purified coagulation factors by Carson (1981). He reported that high-density lipoprotein preparations could inhibit the activation

of tritiated factor X by purified factor VII and tissue factor in a 2-stage radiometric assay system. This result provided the first evidence that lipid could inhibit tissue factor initiated coagulation in a purified protein system.

However, the complexity of the inhibitory mechanism soon unfolded as Dahl et al., (1982) using an amidolytic assay system with tissue factor, purified coagulation factors VII and X observed that the inhibitory effect of normal plasma or serum on the test system was caused by more than one single entity. For example, antithrombin III accounted for only 33% of the inhibitory effect of normal plasma or serum on the test system. In addition, they demonstrated that upon separation of $BaSO_4$-adsorbed plasma into 3 protein peaks according to molecular size, all of which contained inhibitory activities different from antithrombin III; and antithrombin III accounted for only 50% of the inhibitory activity of the third peak.

Finally, in the last 2 years, a series of studies on inhibition of extrinsic pathway of coagulation were published by 3 groups. Rapaport's group first reported that inhibition of tissue factor-factor VIIa activity required factor X and an additional plasma component present in the lipoprotein fraction (Sanders et al., 1985). In a subsequent study, they demonstrated that the simultaneous presence of an apoprotein

preparation rich in apolipoprotein AI, active factor Xa, and Ca^{2+} were required to inhibit the activity of factor VIIa-tissue factor complex in an activation peptide assay using tritiated-factor I-X as substrate (Rao and Rapaport, 1987). Since the inhibition of factor VIIa-tissue factor was reversible, they suggested that the extrinsic pathway inhibitor in the apoprotein preparation exerted its inhibitory effect via binding to a factor VIIa-tissue factor complex formed in the presence of factor Xa and not through degradation of factor VIIa by factor Xa. The requirements of Ca^{2+}, catalytically active factor Xa, and a factor in plasma or serum for tissue factor inhibition were confirmed by both Broze and Miletich (1987) and Hubbard and Jennings (1986) using clotting assays. Hubbard and Jennings (1987) further proposed that apolipoprotein B (in low density lipoprotein) is more important than apolipoprotein A (in high density lipoprotein) in effecting factor Xa associated inhibition of tissue factor-factor VIIa complex. However, the mechanism of inhibition caused by the activated coagulation factors and the lipoproteins was not entirely clear.

In summary, in 1947, Schneider and Thomas demonstrated independently that prior incubation of tissue factor with serum could prevent tissue factor-induced death in mice. Subsequent

experiments *in vitro* revealed that inhibition of tissue factor activity involve at least a multi-component system involving Ca^{2+}, lipoproteins, factor Xa, factor VIIa and tissue factor. The precise mechanism of interactions in this system remains to be elucidated. In addition, experiments to investigate and establish the *in vivo* inhibitory effects of these and other components are still to be performed.

1.4 OBJECTIVES

The initial objective of this thesis was to identify and characterize the components and mechanisms for inhibiting factor VII/VIIa activity. In the process of preparing an inhibitor of the extrinsic pathway of coagulation, an inhibitor of both the extrinsic and intrinsic pathways of coagulation was isolated. This inhibitor was identified as prothrombin F1.2, which could prolong both the prothrombin time and the activated partial thromboplastin time. Thus, the primary objective of this thesis was broadened to investigate the anticoagulant and antithrombotic activities of prothrombin F1.2. The experiments described in this thesis were therefore directed towards :

(i) developing a simple and efficient method for purification of prothrombin F1.2 from plasma

(ii) characterizing the anticoagulant activity of prothrombin F1.2 in normal plasma

(iii) comparing the anticoagulant activity of prothrombin F1.2 in defibrinated plasma to its activity in a purified coagulation factor system

(iv) determining the antithrombotic activity of prothrombin F1.2 in an *in vivo* mouse assay system

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

Units of fresh frozen plasma (plasma prepared from whole blood and processed to the frozen state within 12 h of collection), stored plasma (plasma obtained from whole blood after 24 h storage at 4°C), and cryo-poor plasma (supernant plasma after cryoprecipitate production) were collected and prepared by the Canadian Red Cross Society, Blood Transfusion Service, Hamilton Centre and Toronto Centre.

The human Factor IX Complexes were produced for the Canadian Red Cross Society, Blood Transfusion Service by Connaught Laboratories Ltd., Toronto, Ontario. Pooled normal plasma and congenital factor VII deficient plasma were obtained from George King Bio-Medical, Overland Park, Kansas.

Thromborel S (OUHP 35, a lyophilized lipid-rich extract from human placentas, containing 12.5 mM Ca^{2+}, as a source of tissue factor for prothrombin time assays) was obtained from Behring Canada, Montreal, Quebec. Thromoplastin (rabbit brain acetone powder as a source of tissue factor for prothrombin

time assays) was purchased from Sigma Chemical Company, St. Louis, Missouri. Activated partial thromboplastin reagent (rabbit brain phospholipids with micronized silica as activator) were purchased from General Diagnostics, Toronto, Ontario. Cephalin (6 μg organic phosphate/mL), a chloroform extract of human brain acetone powder, was prepared according to the procedure of Bell and Alton (1954).

Arvin was obtained from Connaught Laboratories Ltd., Toronto, Ontario. One unit of defibrinogenating enzyme corresponds to quantity of enzyme which under standard conditions, defined as 37°C, pH 7.5, produces clotting of 0.1 mL of a 0.3% bovine fibrinogen solution within 240 ± 23 s.

Human α-thrombin (preparation number 301 possessed 2.90 g/L of protein, 2,543 kiloclotting units/g, and an active-site concentration of 1.08×10^{-4} M), and bovine factor Va (0.58 mg/mL) were generously supplied by Drs. J. W. Fenton II, Albany, New York, and C. Esmon, Oklahoma City, Oklahoma, respectively. One clotting unit is that amount of thrombin required to clot 1 mL of a 3.0 g/L standard fibrinogen solution at 37°C in 15 s.

Human factor Xa and thrombin (2,000 kiloclotting units/g) were obtained from Interhematol Inc., Hamilton, Ontario.

S-2222, benzyloxycarbonyl-Ile-Glu-Gly-Arg p-nitroanilide, a chromogenic substrate for factor Xa; S-2238, D-Phe-pipecolyl-Arg p-nitroanilide, a chromogenic substrate for thrombin; S-2251, H-D-Val-Leu-Lys p-nitroanilide dihydrochloride, a chromogenic substrate for plasmin; S-2302, H-D-Pro-Phe-Arg p-nitroanilide djhydrochloride, a chromogeic substrate for plasma kallikrein; plasmin and kallikrein were obtained from Maynard Scientific, Toronto, Ontario.

DEAE-Sepharose, DEAE-Sephadex A-50, Sephacryl S-200, CNBr-activated Sepharose 4B, and benzamidine-Sepharose were purchased from Pharmacia (Canada) Ltd., Montreal, Quebec. Cibacron blue F3GA agarose was obtained from Pierce, Rockford, Illinois. Ultrogel AcA 44 was purchased from Fisher Scientific, Don Mills, Ontario. DEAE-cellulose DE-52 was purchased from Mandel Scientific, Rockwood, Ontario.

Sodium dodecyl sulfate, acrylamide, ammonium persulfate, N,N'-methylene-bis-acrylamide, 2-mercaptoethanol, TEMED, Coomassie brilliant blue R-250, bromophenol blue and molecular weight standards were purchased from Bio-Rad Laboratories, Missisauga, Ontario.

Bovine serum albumin (A-7511, essentially fatty acid free), protein A-alkaline phosphatase conjugate, benzamidine, soybean trypsin inhibitor (Type I-S), carbodiimide (C2388), and

Tris(hydroxymethyl)aminomethane (Tris), diisopropyl fluorophosphate, hypoxanthine, aminopterin, and thymidine were purchased from Sigma Chemical Company, St. Louis, Missouri.

Porcine mucosal heparin (150 USP units/mg) was obtained from Diosynth BV, Oss, The Netherlands. The USP unit of heparin is the quantity that will prevent 1.0 mL of citrated sheep plasma from clotting for 1 h after the addition of 0.2 mL of a 1.0% $CaCl_2$ solution.

IgG fractions of rabbit anti-human IgM and anti-whole human serum were purchased from Cappel, Division of Cooper Biomedicals, Malvern, Pennsylvania.

YM-10 ultrafiltration membranes were purchased from Amicon Canada Ltd. Oakville, Ontario.

Other chemicals, analytical reagent grade, were purchased from BDH Chemicals Canada Limited, Toronto, Ontario.

Balb/c mice and CD1 mice obtained from McMaster University Animal Quarters, Hamilton, Ontario. Goats were obtained from JD Biologicals, Maple, Ontario.

2.2. PREPARATION OF FACTOR VII AND VIIa

Factor VII used in the preparation of murine monoclonal antibodies to factor VII was prepared from human fresh frozen

plasma according to the method of Broze and Majerus (1981) in Fig. 5.

Briefly, 20 L of citrated plasma, to which 156 g benzamidine and 500 mg of soybean tryspin inhibitor had been added, was precipitated with the addition of 1 L of 1.0 M $BaCl_2$. The barium citrate precipitate collected by centrifugation at 3,000 g for 15 min was washed twice with 6 L of 0.2 M NaCl-0.05 M Tris-HCl (pH 8.3)-0.01 M benzamidine, and then resuspended in 3 L of 0.15 M sodium citrate-0.1 M Tris-HCl (pH 8.3)-0.02 M benzamidine and 25 mg/L soybean trypsin inhibitor. The mixture was stirred for 60 min to elute the adsorbed proteins and the barium citrate removed by centrifugation at 3,000 g for 30 min.

The supernatant solution, prepared from barium citrate absorption and elution, was precipitated by the addition of $(NH_4)_2SO_4$ (25% saturation) and the precipitate removed by centrifugation at 6,500 g for 20 min. The supernatant solution was made up to 65% $(NH_4)_2SO_4$ saturation; and the precipitate, collected by centrifugation at 8,500 g for 30 min, was resuspended in 300 mL of 0.2 M EDTA-0.1 M Tris-HCl (pH 8.3)-0.02 M benzamidine and 25 mg/L soybean trypsin inhibitor.

After dialysis of the $(NH_4)_2SO_4$ precipitated material against 0.05 M Tris-HCl (pH 7.5)-0.02 M benzamidine and removal

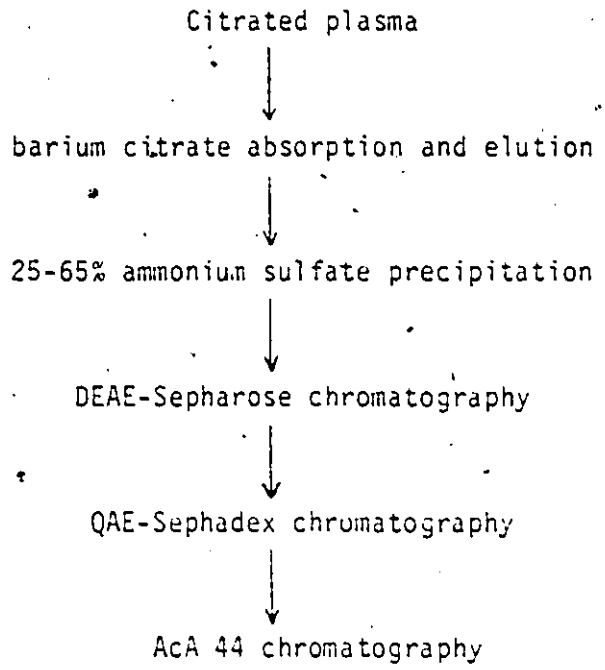


Fig. 5 Purification scheme for factor VII according to the method of Broze and Majerus (1981).

of the precipitate formed during dialysis by centrifugation at 8,500 g for 10 min, the solution was applied to a DEAE-Sepharose column (5 x 35 cm), equilibrated in 0.05 M Tris-HCl (pH 7.5)-0.02 M benzamidine. The column was washed with 1 L of the same buffer and then eluted with a 6 L-linear gradient from 0 to 0.5 M NaCl in 0.05 M Tris-HCl (pH 7.5)-0.02 M benzamidine. The fractions containing factor VII were pooled and the solution was diluted with 50 mM Tris-HCl at pH 7.5 and 20 mM benzamidine to adjust the conductivity to that of 0.15 M NaCl with 50 mM Tris-HCl at pH 7.5 and 20 mM benzamidine.

Six mg of soybean trypsin inhibitor was added to the factor VII pool which was eluted from the DEAE-Sepharose column. The solution was applied to a QAE-Sephadex column (2.5 x 15 cm) equilibrated in 0.15 M NaCl with 50 mM Tris-HCl at pH 7.5 and 20 mM benzamidine. The column was washed with 150 mL of the same buffer and factor VII was eluted with 5 mM $CaCl_2$ in 50 mM Tris-HCl at pH 7.5 and 20 mM benzamidine. The factor VII eluted was collected in 12.5 mM neutralized EDTA and concentrated to 2 mL in an Amicon concentrator with a YM-10 membrane.

The factor VII concentrate, prepared from QAE-Sephadex column chromatography, was applied to a AcA 44 column (1.5 x 95 cm) equilibrated in 0.15 M NaCl, containing 20 mM sodium

phosphate (pH 7.0), 5 mM benzamidine and 1 mM EDTA. The column was washed with the same buffer and the fractions containing factor VII were pooled, concentrated to about 0.5 mg/mL and stored at -70°C.

Factor VII used in the preparation of goat polyclonal antibodies to factor VII and other experiments described in this thesis was prepared by first preparing the 25 to 65% $(NH_4)_2SO_4$ precipitated material as described in the previous paragraphs. However, the dialyzed $(NH_4)_2SO_4$ precipitated material was applied to a monoclonal anti-factor VII Sepharose column (1.6 x 60 cm) instead of DEAE-Sepharose. The monoclonal anti-factor VII Sepharose column was equilibrated in 0.5 M NaCl, containing 50 mM Tris-HCl at pH 7.5 and 20 mM benzamidine. After application of the protein solution, the column was washed with 500 mL of the same buffer and then factor VII was eluted with 200 mL of 0.1M sodium citrate (pH 3.5). The eluate, 8 mL per fraction, was collected into tubes containing 0.4 mL of 1.0 M Tris-HCl (pH 8.5)-0.2 M benzamidine. The protein fractions were pooled and dialyzed against 50 mM Tris-HCl at pH 7.5 and 20 mM benzamidine for 12 h with 3 changes of dialysis buffer of 4 L each.

The dialyzed material, after monoclonal anti-factor VII Sepharose column chromatography, was applied to an anti-IgM

Sepharose column (1.0 x 15 cm) equilibrated in 50 mM Tris-HCl (pH 7.5) and 20 mM benzamidine. The column was washed with the same buffer and the unadsorbed protein collected was applied to an anti-whole human serum Sepharose column (1.6 x 60 cm) equilibrated in 0.05 M Tris-HCl and 20 mM benzamidine (pH 7.5). The column was washed with the same buffer, unadsorbed protein, purified factor VII, were pooled, concentrated to 0.5 mg/mL (Amicon YM-10) and stored at -70°C.

Factor VIIa was prepared from stored human plasma as described previously for preparation of factor VII using affinity chromatography except the use of benzamidine and soybean trypsin inhibitor was omitted in all steps.

2.3. PREPARATION OF MURINE MONOCLONAL ANTIBODY TO HUMAN FACTOR VII

The strategy used in the production of monoclonal antibody was that reported by Kohler and Milstein (1975). Briefly, BALB/c mice were injected subcutaneously with 50 ug of human factor VII (prepared by the method described in Section 2.2.) in 0.5 mL saline mixed with an equal volume of Freund's complete adjuvant, followed by two additional weekly injections of 50 ug each in Freund's incomplete adjuvant. The presence of

anti-factor VII antibody was detected using an enzyme immuno assay (Section 2.4.). The mouse with the highest antibody level was used for fusion. Three days before fusion, the mouse was injected intraperitoneally with an additional 100 ug of factor VII with no adjuvant.

On the day of cell fusion, the spleen from the immunized mouse was removed asceptically and washed with serum-free Iscoves Modified Dulbecco's Medium (IMDM). A 15 to 20 mL cell suspension was made by mincing the spleen through a sterile steel screen. The cell suspension was centrifuged at 250 g for 10 min at room temperature. The spleen cells were treated for 5 min with 2 mL of NH_4Cl solution, containing 0.8 g NH_4Cl, 0.1 g $KHCO_3$, and 0.1 mM Na_2EDTA (pH 7.1) in 100 mL to lyse the red cells. The cell suspension was then made up to 12 mL with serum-free IMDM, and 10 µL was used to assess the cell density on a Coulter Counter Model S. Myeloma P3-NS 1 cells, in exponential growth phase, suspended in serum-free IMDM was counted on a Coulter Counter Model S. The volumes of both spleen cell and myeloma cell suspensions were adjusted to give 10^8 spleen cells and 10^7 myeloma cells each in 2 mL. Equal volumes of each cell suspension were combined. The mixture was centrifuged at 250 g for 10 min and the supernatant solution removed. Then, 0.5 mL fusion solution (0.25 mL

dimethylsulfoxide, 2.75 mL IMDM, 2.0 mL polyethlene glycol 1,000 initiated to pH 7.4 with 7% $NaHCO_3$) was added slowly over 1 min, with agitation to the loosened cell pellet. The tube containing the cell suspension was placed into a 37°C water bath and swirled for 1 min. Ten mL serum-free IMDM was added slowly over 2 min, to the cell suspension. The tube was then placed at room temperature for 5 min, followed by centrifugation at 200 g for 10 min. The cell pellet was gently resuspended in 10 mL serum-free IMDM and centrifuged at 200 g for 10 min. The cells were resuspended in the HAT selection medium (IMDM containing 10% fetal calf serum, 10% penicillin streptomycin, and 2% HAT) at a concentration of 10^5 myeloma cells/mL and plated into 96 well-plates with 0.2 mL per well. The plates were incubated in a CO_2 incubator.

Growth of hybrid cells was evident after 10 to 15 days incubation, as the medium turned yellow. The supernatant solutions were screened for antibodies to factor VII using an enzyme immuno assay (Section 2.4.). Cells secreting antibodies to factor VII were then cloned by limiting dilution.

For production of large quantities of monoclonal antibody, BALB/c mice were primed with 0.5 mL of pristane administered 2 weeks prior to intraperitoneal injection of 10^6 hybrid cells in 0.1 mL. Tumor growth was evident in about 2 to

3 weeks, then the mice were killed and the ascites fluid, about 5 mL per mouse, was drained from the peritoneum.

2.4. ENZYME IMMUNO ASSAY FOR DETECTION OF MURINE ANTIBODY TO HUMAN FACTOR VII

Sera from mice immunized with human factor VII or culture supernatants from hybridoma cell lines were assayed for antibody to factor VII based on the enzyme immuno assay of Cerskus et al. (1985). Polyvinyl chloride microtitre plates were activated with 200 uL of carbodiimide (10 mg/mL in 0.1 M sodium carbonate buffer, pH 9.6) per well. After overnight incubation at 4°C the wells were washed with PBS-Tween (0.05% Tween 20 in 0.15 M NaCl, 0.1 M phosphate, pH 7.4). The wells were coated with 200 µL purified factor VII (500 ng/well) by incubation at 37°C for 1 h and washed. The following additions were made sequentially, each incubated at 37°C for 1 h and then washed : 200 µL of mouse serum or hybridoma supernatant or ascites fluid or 1.0% bovine serum albumin in PBS-Tween for blank wells; 50 µL of rabbit anti-mouse IgG; 200 µL of protein A-alkaline phosphatase conjugate. Finally, 200 µL of p-nitrophenyl phosphate (1.5 mg/mL) was added, incubated for 1 h at 37°C and the reaction stopped by the further addition of

20 µL of 5 N NaOH. The plates were read at 405 nm on a Titertek Multiscan plate reader. Dilutions yielding absorbance readings 0.2 absorbance units above the blank wells were used to determine the titres of antibody in the serum or ascites fluid.

2.5. PREPARATION OF POLYCLONAL ANTIBODIES

For the production of antibodies to human factor VII and prothrombin F1.2, goats were immunized with 500 µg of purified protein in saline mixed with an equal volume of Freund's complete adjuvant. The same immunization dose was used twice at two week intervals after the initial immunization to booster the production of antibodies. Blood was collected 2 weeks later. The presence of antibody against the specific antigen was assessed by double diffusion in agarose (Section 2.13.).

2.6. PREPARATION OF IgG FRACTION OF MONOCLONAL AND POLYCLONAL ANTIBODIES

IgG fraction of murine monoclonal anti-factor VII was prepared from ascites fluid from mice, in which hybridoma cells

were grown as ascites tumors (Cerskus et al., 1985). About 5 to 7 mL of ascites fluid was obtained from each mouse, and centrifuged at 10,000 g for 3 min. The supernatant solution was applied to a 5 mL column of protein A-Sepharose previously equilibrated with 0.1 M phosphate buffer (pH 8.0). The column was then eluted successively with 40 mL each of 0.1 M sodium citrate (pH 6.0); 0.1 M sodium citrate (pH 4.5); and 0.1 M sodium citrate (pH 3.5). Fractions were monitored for the presence of anti-factor VII antibody by enzyme immuno assay, and anti-factor VII was observed in the 0.1 M sodium citrate (pH 3.5) eluate. The fractions containing anti-factor VII were pooled, dialyzed overnight against 3 changes of 4 L of 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.5 M NaCl and kept frozen at -30°C.

The IgG fraction of goat polyclonal anti-factor VII was prepared according to the method published by Pharmacia Fine Chemicals. Briefly, 25 mL of goat antiserum was made to 50% saturation with the addition of $(NH_4)_2SO_4$. The precipitate obtained by centrifugation at 8,000 g for 30 min was dissolved in an ethylenediamine buffer (3.2 mL ethylenediamine and 4.2 mL glacial acetic acid in 1 L, pH 7.0, ionic strength of 0.1 M). The protein solution was dialyzed against 4 changes of 4 L of ethylenediamine buffer.

The dialyzed solution, after $(NH_4)_2SO_4$ precipitation, was applied to a column of 100 mL of QAE-Sephadex A-50 equilbrated with the same buffer. The same ethylenediamine buffer was used to elute the goat IgG from the column. The eluted goat anti-factor VII was pooled, dialyzed against 3 changes of 4 L of 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.5 M NaCl and kept frozen at -30°C. The QAE-Sephadex A-50 was regenerated with an acetate buffer containing 14.9 mL of glacial acetic acid and 10.6 g of sodium acetate in 1 L (pH 4.0, ionic strength 0.1 M).

2.7. PREPARATION OF GROUP SPECIFIC SEPHAROSE

IgG fractions of murine monoclonal anti-factor VII, goat polyclonal anti-factor VII, goat anti-human IgM, and goat anti-whole human serum were coupled to CNBr-activated Sepharose 4B by the method described by Pharmacia Fine Chemicals.

The required amount of freeze-dried CNBr-activated Sepharose 4B was swollen (1 g freeze-dried powder gives about 3.5 mL of gel) for 15 min in 1 mM HCl and washed on a G3 porosity sintered glass filter with the same buffer. A total of about 200 mL/g dry gel was washed through in several aliquots. The gel was then washed with coupling buffer (5 mL/g

dry gel) and immediately transferred to the protein solution in coupling buffer. 0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl was used as the coupling buffer, and about 5 mg protein was used for each mL of gel in a gel to buffer ratio of 1:2. The protein-gel suspension was mixed in a end-over-end mixer overnight at 4°C. The protein solution was removed by centrifugation at 500 g for 10 min. The gel was transferred to 0.2 M glycine, pH 8.0 and mixed for 16 h at 4°C. The gel was then washed alternatively with 0.5 M NaCl buffered with 0.1 M acetate buffer at pH 4.0 and coupling buffer four or five times. The specific protein-Sepharose conjugate was stored at 4°C.

2.8. INITIAL ISOLATION AND CHARACTERIZATION OF PROTHROMBIN FRAGMENT 1.2

A simple overview of the steps involved in initial isolation of prothrombin F1.2 is shown in Fig. 6.

Ten L of cryo-poor human plasma was absorbed with 400 g DEAE-cellulose (DE-52) by mixing for 2 h and overnight settling at 4°C. The mixture was centrifuged at 5,000 g for 20 min, and supernant plasma discarded. The DEAE-cellulose was washed twice with 500 mL 0.03 M citrate buffer (pH 6.8); and adsorbed

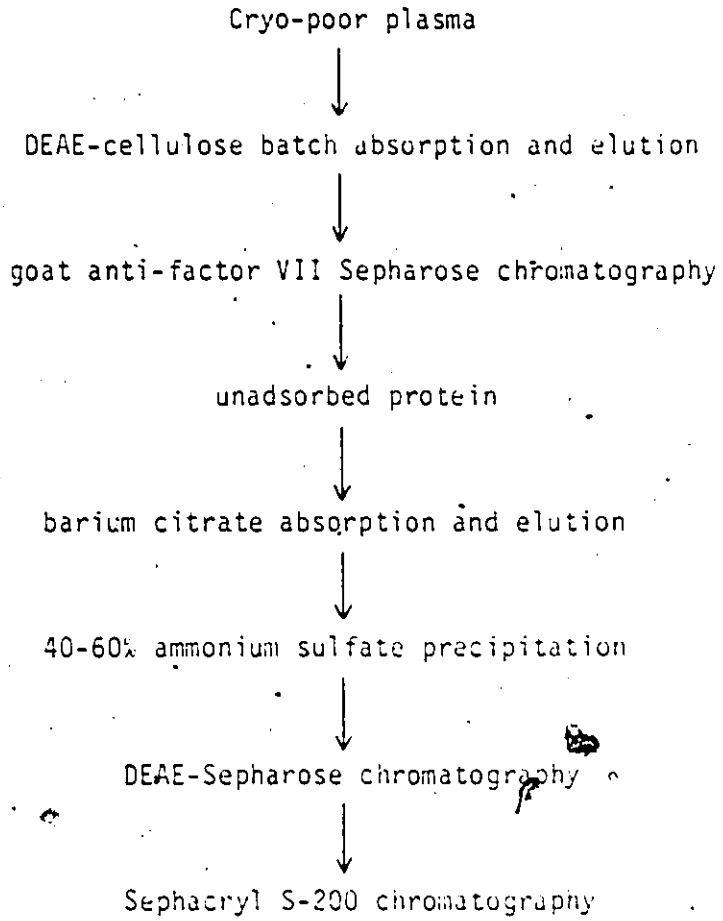


Fig. 6 Initial isolation of prothrombin fragment 1.2.

proteins were eluted with 500 mL 0.5 M NaCl buffered with 0.01 M sodium phosphate buffer at pH 7.0. The eluted material was dialyzed overnight against 3 changes of 4 L 0.15 M NaCl and 50 mM Tris-HCl at pH 7.5.

The protein solution, obtained from DEAE-cellulose absorption and elution, was applied to a column of goat anti-factor VII Sepharose (2.6 x 35 cm) equilibrated in 0.15 M NaCl buffered with 50 mM Tris-HCl at pH 7.5. The unadsorbed protein was pooled and tested in a prothrombin time assay system to assess the clotting time compared to a buffer control.

Sodium citrate (8.9 g) was added to the unadsorded protein pool which did not adsorbed to the goat anti-factor VII Sepharose column. This was followed by dropwise addition of 60 mL of 1.0 M $BaCl_2$ to the protein pool. The mixture was stirred for 1 h. The barium citrate precipitate was collected by centrifugation at 3,000 g for 15 min. The precipitate was washed twice with 500 mL of 0.2 M NaCl buffered with 50 mM Tris-HCl at pH 8.5, and resuspended in 500 mL of 0.15 M sodium citrate buffered with 0.05 M Tris-HCl at pH 8.5. The mixture was stirred for 1 h to elute adsorbed proteins, and the barium citrate removed by centrifugation at 3,000 g for 30 min.

After barium citrate absorption and elution, $(NH_4)_2SO_4$

was added to bring the solution to 40% saturation, and the precipitate removed by centrifugation at 6,500 g for 20 min. $(NH_4)_2SO_4$ was added slowly to the supernant solution to take the $(NH_4)_2SO_4$ concentration to 60% saturation. The precipitate was collected by centrifugation at 8,500 g for 30 min, resuspended in 0.05 M Tris buffered with HCl at pH 7.5, and dialyzed against the same buffer with 3 changes of dialysis buffer of 4 L each.

The dialyzed material, obtained after $(NH_4)_2SO_4$ precipitation, was applied to a DEAE-Sepharose column (2.6 x 35 cm) equilibrated with 0.05 M Tris-HCl at pH 7.5. The column was washed with 300 mL of the equilibrating buffer and then eluted with 1,500 mL-linear gradient from 0 to 0.5 M NaCl in 50 mM Tris-HCl at pH 7.5. The fractions were tested in a prothrombin time assay system to assess the clotting time compared to a buffer control. Fractions (85 to 95), which gave a prolonged clotting time were pooled, and dialyzed against 3 changes of 0.15 M NaCl buffered with 50 mM Tris-HCl at pH 7.5 of 4 L each.

The dialyzed material, obtained after DEAE-Sepharose column chromatography, was concentrated to 6.0 mL and applied to a column (2.6 x 35 cm) of Sephacel S-200 in 0.15 M NaCl-50 mM Tris-HCl at pH 7.5. The fractions were tested in a

prothrombin time assay system, and fractions (28 to 32) which gave a prolonged clotting time were pooled and stored at -30°C.

The purified protein was dialyzed overnight against 2 changes of 4 L of distilled deionized water. Ultracentrifugation was performed with a Beckman Model E Analytical Ultracentrifuge with UV. optics at 280 nm and 20°C. Amino acid analysis was performed, after 20-h hydrolysis with 5.7 N HCl at 107°C in evacuated tubes, using a Beckman 120C Amino Acid Analyzer. Sequencing was performed with an Applied Biosystems Model 470A Gas Phase Sequenator coupled to a Model 120 PTH Amino Acid Analyzer. Amino acid analysis, sequencing, and ultracentrifugation were performed by Max Blum and Charles Yu of University of Toronto, Toronto, Ontario.

2.9. PREPARATION AND CHARACTERIZATION OF PROTHROMBIN FRAGMENT 1.2

A simple flowchart for the preparation of prothrombin F1.2 is shown in Fig. 7.

Stored plasma, typically 4 L was warmed to 37°C in a glass beaker. The plasma was converted to serum by the addition of 40 mL of a 1.0 M $CaCl_2$ solution. Fibrin formation was apparent within 15 min after the addition of $CaCl_2$. The

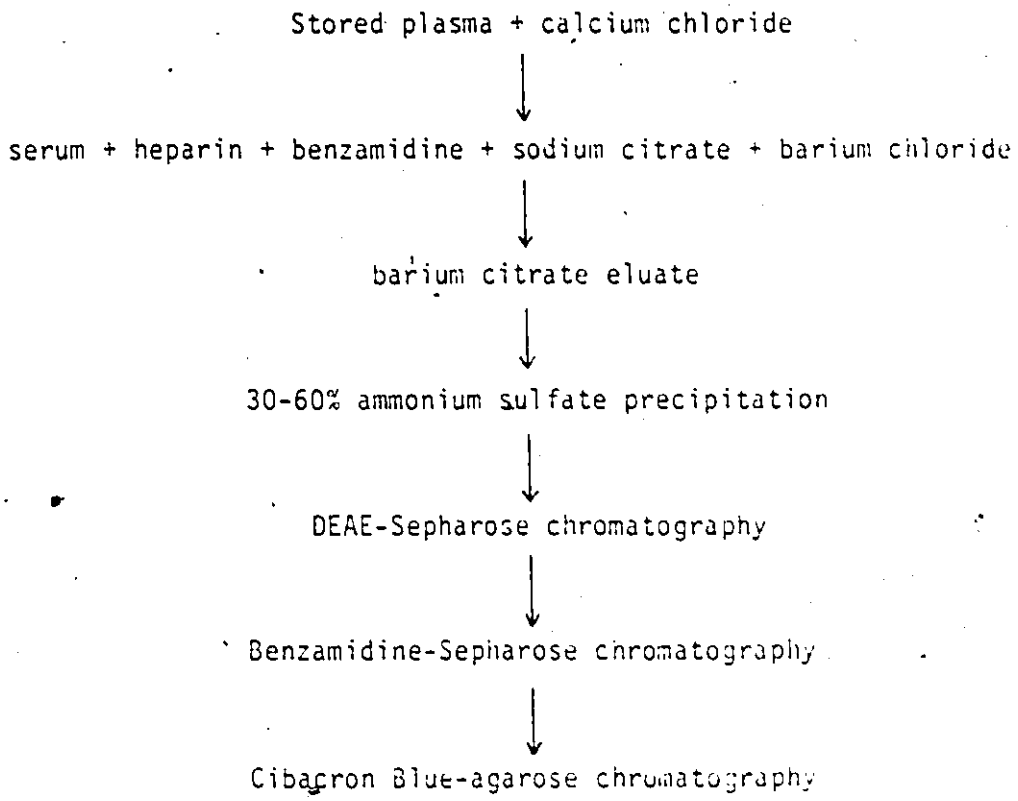


Fig. 7 Purification scheme for prothrombin fragment 1.2.

plasma was allowed to clot and the clot was wound with a glass rod. Small strands of fibrin, if present were removed by centrifugation at 3,000 g for 5 min.

At 2 h after the addition of $CaCl_2$, 4,000 units of heparin was added to the serum to enhance the inactivation of thrombin by antithrombin III. Following the addition of heparin, 31.2 g benzamidine (0.05 M) were added to inhibit activation of residual coagulation factors and activities of residual activated coagulation factors present in the serum. The serum was transferred to the cold room and all subsequent steps were performed at $4^{o}C$.

Sodium citrate (35.3 g) was then added to the serum followed by dropwise addition of 240 mL of 1.0 M $BaCl_2$. The mixture was stirred for a total of 1 h. The barium citrate precipitate was collected by centrifugation at 3,000 g for 15 min and the supernatant serum decanted. The precipitate was washed twice with 1 L of 0.2 M NaCl-0.05 M Tris-HCl (pH 8.5)-0.02 M benzamidine, and then resuspended in ½ L of 0.15 M sodium citrate-0.05 M Tris-HCl (pH 8.5) containing 0.02 M benzamidine. The mixture was stirred for 60 min to elute adsorbed proteins, and the barium citrate removed by centrifugation at 3,000 g for 30 min.

After barium citrate absorption and elution, $(NH_4)_2SO_4$

(123 g) was added to the supernatant solution (700 mL) slowly with stirring (30% saturation), and the precipitate removed by centrifugation at 6,500 g for 20 min. An additional 145 g of $(NH_4)_2SO_4$ was added slowly to the supernatant solution (730 mL) with stirring (60% saturation). The precipitate was collected by centrifugation at 8,500 g for 30 min, resuspended in 0.1 M NaCl-0.05 M Tris-HCl (pH 7.5)-0.02 M benzamidine, and dialyzed against the same buffer for 12 h with 3 changes of dialysis buffer of 4 L each. The precipitate developed during dialysis was removed by centrifugation at 8,500 g for 10 min.

The dialyzed material, after $(NH_4)_2SO_4$ precipitation, was applied to a DEAE-Sepharose column (2.6 x 35 cm) equilibrated with 0.1 M NaCl, 50 mM Tris-HCl at pH 7.5, and 20 mM benzamidine, at a flow rate of 35 mL/h. The column was washed with 300 mL of the equilibrating buffer and then eluted with 1 L-linear gradient from 0.1 to 0.5 M NaCl containing 0.05 M Tris-HCl at pH 7.5 and 0.02 M benzamidine. Prothrombin F1.2 was detected by double diffusion in agarose gel with a goat antibody, and eluted at an ionic strength of about 0.25 M NaCl. The fractions (52 to 58) containing F1.2 were pooled and dialyzed against 4 changes of 90 min intervals of 4 L of 0.05 M Tris-HCl at pH 7.5.

The dialyzed material, obtained from DEAE-Sepharose

column chromatography, was applied to a benzamidine-Sepharose column (1.5 x 28 cm) equilibrated with 0.05M Tris-HCl at pH 7.5. The unadsorbed protein was pooled and applied to a Cibacron Blue-agarose column (1.5 x 28 cm) equilibrated with 0.05 M Tris-HCl at pH 7.5. The unadsorbed protein was pooled and dialyzed against 4 changes at 6-h intervals of 4 L of 0.15 M NaCl-0.05 M Tris-HCl (pH 7.5). The dialyzed material was kept frozen at -30°C.

Amino acid analysis, amino acid sequence and ultracentrifugation were performed as described in the previous section 2.8.

Experiments to assess amidolytic activities of F1.2 and effects of F1.2 on amidolytic activities of factor Xa and thrombin were performed with different chromogenic substrates. In experiments to assess amidolytic activities, 0.5 mL of a 2 mg/mL F1.2 solution was added to 0.5 mL chromogenic substrate (1 mM S-2222, S-2238, S-2251, or S-2302). Positive controls were set up by adding 0.5 mL of 20 µg/mL factor Xa, 20 µg/mL thrombin, 25 casein units/mL plasmin and 0.6 units/mL kallikrein to 0.5 mL 1 mM S-2222, S-2238, S-2251, and S-2302, respectively. Negative controls were set up by adding 0.5 mL Tris-buffered saline to 0.5 mL of 1 mM chromogenic substrates. After 24 h incubation at 37°C, the reaction mixtures were read

against their respective negative controls as blanks in a Gilford spectrophotometer. In experiments to assess effect of F1.2 on amidolytic activity of factor Xa, reaction mixtures were made up of 150 µL of factor Xa (3.6 µg/mL) and 150 µL of F1.2 (2 mg/mL) or Tris-buffered saline and 500 µL of 1 mM S-2222. After 1 min incubation at 37°C, 200 µL of 50% acetic acid was added to each reaction mixture. The absorbance at 405 nm for each reaction mixture was read in a Gilford Response spectrophotometer. In experiments to assess effect of F1.2 on amidolytic activity of thrombin, reaction mixtures were made up of 150 µL of thrombin (50 pmole active site thrombin/mL) and 150 µL of F1.2 (2 mg/mL) or Tris-buffered saline and 500 µL of 500 µM S-2238. After 1-min incubation at 37°C, 200 µL of 50% acetic acid was added to each reaction mixture. The absorbance at 405 nm for each reaction mixture was read in a Gilford Response spectrophotometer.

2.10. PREPARATION OF HUMAN PROTHROMBIN AND FACTOR X

Human prothrombin and factor X were prepared from Factor IX Complex (supplied by Connaught Laboratories) as described by Modi et al. (1984), Fig. 8.

Briefly, 9 vials of human Factor IX Complex were

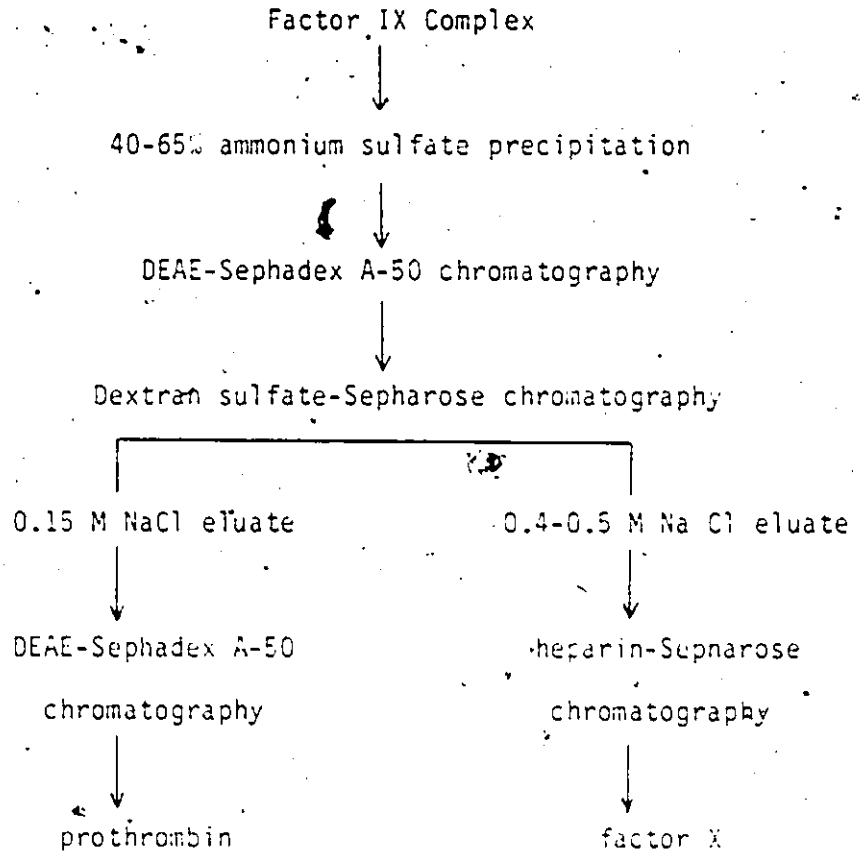


Fig. 8 Purification scheme for prothrombin and factor X.

dissolved in 500 mL of a buffer containing 0.025 M sodium citrate (pH 6.0), 1.0 mM DIP-F, 10 mM benzamidine, soybean trypsin inhibitor (10 mg/L) and heparin (1,000 units/L). After stirring for 15 min, the solution was brought to 40% saturation with the addition of $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 5,000 g for 20 min. The supernatant solution was then brought to 65% saturation with addition of $(NH_4)_2SO_4$. The precipitate collected by centrifugation at 5,000 g for 20 min was dissolved in 50 mL of 25 mM citrate (pH 6.0) containing 1 mM DIP-F, and 10 mM benzamidine and dialyzed overnight against 4 changes of the citrate solution. After removing the precipitate by centrifugation at 8,000 g for 10 min, the dialyzed material was applied to a DEAE-Sephadex A-50 column (2.5 x 35 cm) equilibrated in the citrate buffer. The column was washed with 1 L of the same buffer containing 0.1 M NaCl. Protein was eluted with 800 mL of a linear gradient of 0.1 to 0.6 M NaCl in the equilibrating citrate buffer. The fractions containing prothrombin and factor X were pooled and dialyzed against two changes of 4 L of 0.014 M sodium citrate at pH 7.4 containing 10 mM benzamidine and 0.5 mM DIP-F.

The dialyzed material, obtained after DEAE-Sephadex column chromatography, was applied to a dextran sulfate-Sepharose column (2.5 x 35 cm) equilibrated in 0.014 M

sodium citrate at pH 7.4 containing 10 mM benzamidine and 0.5 mM DIP-F. The column was washed with 150 mL of the same buffer containing 0.1 M NaCl, and prothrombin was eluted with 500 mL of the same buffer containing 0.15 M NaCl. Other proteins were eluted with 600 mL of a linear gradient of 0.15 to 0.7 M NaCl in the equilibrating citrate buffer. Factor X eluted at 0.4 to 0.5 M NaCl.

The fractions containing prothrombin were pooled and dialyzed overnight against 2 changes of 0.025 M sodium citrate at pH 6.0 containing 0.5 mM DIP-F and 2 mM benzamidine. The dialyzed material was applied to a second DEAE-Sephadex A-50 column (2.5 x 35 cm) equilibrated with the citrate solution. Prothrombin was separated from other proteins by eluting with 500 mL of a linear gradient from 0.05 to 0.6 M NaCl in the same buffer. The fractions containing purified prothrombin were pooled, dialyzed against 3 changes of 4 L each of Tris-buffered saline and stored in 50% glycerol at -20°C.

The fractions containing factor X eluted from the dextran sulfate-Sepharose column were pooled and dialyzed overnight against 2 changes of 0.05 M NaCl containing 0.05 M Tris-HCl at pH 7.45 and 2 mM benzamidine. After dialysis, $CaCl_2$ was added to the dialyzed material to a $CaCl_2$ concentration of 1 mM. The solution was applied to a

heparin-Sepharose column (1.6 x 8.5 cm) equilibrated with 50 mM NaCl containing 50 mM Tris-HCl at pH 7.45, 2 mM benzamidine, and 1 mM $CaCl_2$. The column was washed with 30 mL of the same buffer. Factor X was eluted by the application of 300 mL linear gradient from 0.2 to 0.7 M NaCl in 1 mM $CaCl_2$ buffered with 50 mM Tris-HCl (pH 7.45) containing 2 mM benzamidine. The fractions containing factor X were pooled, dialyzed against 3 changes of 4 L each of Tris-buffered saline and stored in 50% glycerol at -20°C.

2.11. PREPARATION OF DEFIBRINATED PLASMA

In order to eliminate clotting of plasma subsequent to the activation of factor X or prothrombin, pooled normal plasma was defibrinated with 0.15 units of Arvin per mL of plasma. This was accomplished by the addition of 5 µL of Arvin (30 units/mL) to 1.0 mL of plasma. Subsequent to the addition of Arvin, the plasma was allowed to clot for 10 min at 37°C. The clot was removed with a wooden applicator stick and the sample was reincubated for 5 min. Any residual fibrin clot was similarly removed, and wisps of fibrin were removed by centrifugation at 5,000 g for 2 min. The defibrinated plasma was stored at 4°C and used within 60 min. No further clotting

of the plasma was observed during its use.

2.12. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-polyacrylamide gel electrophoresis was performed in discontinuous polyacrylamide gels composed of an upper stacking gel of 4% acrylamide and a lower resolving gel of 12% acrylamide. The gels were prepared in the buffer system of Laemmli (1970). The 4% stacking gel was prepared by mixing 12.2 mL of water, 5.0 mL of 0.5 M Tris-HCl, pH 6.8, 0.2 mL of 10% (w/v) SDS solution, 2.6 mL of 30% (w/v) acrylamide/bisacrylamide solution, 0.1 mL of 10% (w/v) ammonium persulfate solution, and 0.02 mL of TEMED. The 12% resolving gel was prepared by mixing 33.5 mL of water, 25.0 mL of 1.5 M Tris-HCl, pH 8.8, 1.0 mL of 10% (w/v) SDS solution, 40.0 mL of 30% (w/v) acrylamide/bisacrylamide solution, 0.5 mL of 10% (w/v) ammonium persulfate, and 0.05 mL of TEMED.

Protein samples for electrophoresis were mixed with an equal volume of sample buffer containing 0.125 M Tris-HCl at pH 6.8, 4% (w/v) of SDS, and 20% (v/v) of glycerol. The mixture was heated for 3 min in a boiling waterbath, and afterwards kept on ice until applied to the wells for electrophoresis. The same procedure was used for electrophoresis of reduced

proteins, except an additional 10% (v/v) of 2-mercaptoethanol was present in the sample buffer.

Electrophoresis was performed in 1.5-mm thick, 16-cm wide, 16-cm long gels in a Bio-Rad Protean II slab cell electrophoresis apparatus. Electrode buffer containing 3.0 g/L of Tris, 14.4 g/L of glycine, and 1.0 g/L of SDS at pH 8.3 was used to fill both the upper and lower chambers. The gels were electrophoresed under constant current conditions at 25 mA per gel with a Bio-Rad Model 500/200 power supply unit.

After electrophoresis for 5 h, the gels were stained for 1 h with 0.1% Coomassie Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid. The gels were destained for 12 h with 40% (v/v) methanol and 10% (v/v) acetic acid in a Bio-Rad Model 556 gel destainer to remove background.

2.13. IMMUNOELECTROPHORESIS AND IMMUNODIFFUSION

The method of Grabar and Williams (1955) was used for immunoelectrophoresis in 1.0% agarose. The buffer was barbital buffer at pH 8.6, and ionic strength 0.075 M (3.0 g 5,5-diethylbarbituric acid and 15.5 g 5,5-diethyl-sodium barbiturate in 1.0 L water). Electrophoresis was performed at 10 volts/cm for 1 h. Antibody troughs were cut and filled with

100 uL of antiserum. After development in a humidified chamber at room temperature for 16 h, plates were washed for 24 h with two changes of 0.9% NaCl and 8 h in water. The plates were stained for 10 min with 0.1% amidoblack in 40% (v/v) methanol and 10% (v/v) acetic acid, the destain solution was made up of 40% (v/v) methanol and 10% (v/v) acetic acid.

Immunodiffusion was performed according to the method of Ouchterlony (1968) in 1.0% agarose in barbital buffer at pH 8.6 and ionic strength 0.075 M. Double diffusion was allowed to proceed for 16 h in a humidified chamber at room temperature. Plates were washed, stained, and destained as described for immunoelectrophoresis.

2.14. IN VITRO ASSAYS

2.14.1. PROTHROMBIN TIME

Clotting time for tissue factor initiated coagulation was determined with a BBL fibrometer. 75 µl of pooled normal plasma and 75 µl of F1.2 or Tris-buffered saline were incubated at 37°C. After 3 min incubation, 150 µl of prewarmed Thromborel S was added, and the clotting time recorded. Different concentrations of F1.2 were obtained by dilution with

Tris-buffered saline. For experiments involving different dilutions of plasma, the dilutions were performed with Tris-buffered saline. Thromborel S was diluted with 12.5 mM $CaCl_2$ for experiments involving different concentrations of tissue factor.

2.14.2. ACTIVATED PARTIAL THROMBOPLASTIN TIME

Clotting time for coagulation initiated with activated partial thromboplastin reagent was determined with a BBL fibrometer. The assay was performed by first incubating 100 µL of pooled normal plasma and 100 µL of F1.2 or Tris-buffered saline with 100 µL of activated partial thromboplastin reagent for 3 min at 37°C, and coagulation was started with the addition of 100 µL of prewarmed 25 mM $CaCl_2$.

2.14.3. ASSAY OF INHIBITION OF TISSUE FACTOR ACTIVITY

Particulate tissue factor was prepared from Sigma rabbit brain thromboplastin as follows : The content of 1 vial of thromboplastin (40 mg rabbit brain acetone powder) was reconstituted with 2.0 mL of Tris-buffered saline containing 25 mM EDTA. The content of the vial was centrifuged for 10 min in

an Eppendorf microcentrifuge Model 5414. The pellet obtained was washed 4 times with 2.0 mL of Tris-buffered saline containing 25 mM EDTA, 5 times with 2.0 mL of Tris-buffered saline without EDTA, and 2 times with Tris-buffered saline containing 3 mM $CaCl_2$. The washed tissue factor pellet was resuspended in 2.0 mL of Tris-buffered saline containing 3 mM $CaCl_2$.

Dialyzed defibrinated plasma was prepared by dialyzing citrated plasma overnight against Tris-buffered saline, and defibrinated with Arvin as described in Section 2.11.

The effect of various components on tissue factor activity was assessed by first incubating at 37°C, 125 µL of washed tissue factor suspended in 3 mM $CaCl_2$ in Tris-buffered saline with 125 µL of (i) F1.2, (ii) F1.2 and factor VIIa, (iii) F1.2, factor VIIa and factor Xa, (iv) Tris-buffered saline, (v) Tris-buffered saline and factor VIIa, (vi) Tris-buffered saline, factor VIIa and factor Xa, (vii) dialyzed defibrinated plasma. The total volume of the incubation mixture was maintained at 250 µL with Tris-buffered saline, and the concentration of $CaCl_2$ kept at 1.5 mM. The concentrations of F1.2, factor VIIa and factor Xa when present in the reaction mixtures were 50 µg/mL, 50 ng/mL, and 1.5 µg/mL respectively. Then after 1 h incubation at 37°C, 1.0 mL of Tris-buffered

saline containing 1.5 mM $CaCl_2$ was added to each reaction mixture, and the mixtures were centrifuged in an Eppendorf microcentrifuge Model 5414 for 10 min. Each tissue factor pellet was resuspended in 250 µL of 12.5 mM $CaCl_2$. The tissue factor activity of each pellet was assayed by determining the clotting time of 0.2 mL resuspended pellet and 0.1 mL pooled normal plasma.

2.14.4. AMIDOLYTIC ASSAY FOR FACTOR X ACTIVATION

The amidolytic assay for the generation of factor Xa using the chromogenic substrate S-2222 in defibrinated plasma and in purified protein system was a modification of the method from Hultin (personal communication). Tris-buffered saline containing 0.1 mg/mL of bovine serum albumin was used as a diluent for the purified proteins and as the buffer referred in this study. A supernatant solution prepared by centrifugation of Thromborel S at 10,000 g for 15 min was used as tissue factor.

In the defibrinated plasma system, 50 µL of defibrinated plasma, 50 µL of factor X (50 µg/mL) and 50 µL of buffer or F1.2 (365 µg/mL) were incubated in a 12 x 75-mm plastic tube at 37°C. After 5 min incubation, 50 µL of tissue

factor also preincubated at 37°C for 5 min was added. Then, at specific times, 50 µL of 0.15 M neutralized EDTA was added, and the tube was placed on ice.

In the purified protein system, 50 µL of factor VIIa (3 µg/mL), 50 µL of tissue factor, and 50 µL of buffer or F1.2 (365 µg/mL) were incubated in a 12 x 75-mm plastic tube at 37°C. After 5 min incubation, 50 µL of factor X (50 µg/mL) also preincubated at 37°C for 5 min was added. At specific times, 50 µL of 0.15 M neutralized EDTA was added, and the tube was placed on ice.

The amount of factor Xa produced in either the defibrinated plasma or purified protein system was quantitated by the amidolysis of S-2222 by factor Xa at 405 nm. This was accomplished by subsampling 200 µL of the factor X reaction mixtures into microcuvettes positioned in a Gilford Response spectrophotometer equipped with a thermal heating cuvette assembly set at 37°C. The cuvettes contained 200 µL of 1 mM S-2222. The cuvettes and their contents had been preincubated at 37°C for 5 min before the addition of the factor X reaction mixtures. The initial rate change was determined from change in absorbance at 405 nm monitored over 2 min. The concentration of factor Xa generated in the reaction mixture was calculated from initial rate change obtained with known

concentrations of purified factor Xa determined under the same condition as described above. For example, the initial rate change associated with 1 nM of purified factor Xa was 0.02 absorbance unit in 1 min. This was determined by sampling 200 µL from a mixture containing 200 µL of 1 nM factor Xa and 50 µL of 0.15 M neutralized EDTA into 200 µL of 1 mM S-2222.

2.14.5. ASSAY OF ACTIVATION OF PROTHROMBIN IN DEFIBRINATED PLASMA

The activation of plasma prothrombin in defibrinated plasma was quantitated by assessing the amidolytic activity of thrombin on chromogenic substrate S-2238 in a 2-stage assay. For assessment of thrombin generation via the extrinsic pathway, 100 µL of defibrinated plasma was added to 100 µL of Tris-buffered saline or 100 µL of F1.2 dilutions in a 12 x 75-mm plastic tube incubated at 37°C. After a 3-min incubation, 100 µL Thromborel S also preincubated at 37°C for 3 min was added. Then at specific times, 50 µL of reaction mixture was subsampled into a plastic tube, containing 200 µL 5 mM neutralized EDTA in an ice bath.

For assessment of thrombin generation via intrinsic pathway, 100 µL of defibrinated plasma and 50 µL of activated

partial thromboplastin reagent were added to 100 µL of Tris-buffered saline or 100 µL of F1.2 dilutions in a 12 x 75 mm plastic tube at 37°C. After 3 min incubation, 50 µL of 25 mM $CaCl_2$ also preincubated at 37°C for 3 min was added. At specific times, 50 µL of reaction mixture was subsampled into a plastic tube, containing 200 µL of 5 mM neutralized EDTA in an ice bath.

The amount of thrombin generated by either the extrinsic or intrinsic pathway was quantitated by the amidolysis of S-2238 by thrombin at 405 nm. This was accomplished by subsampling 25 µL of EDTA-reaction mixture into 775 µL of 200 µM S-2238 prewarmed at 37°C for 3 min. Then at exactly 1 min after the addition of the EDTA-reaction mixture, 200 µL of 50% acetic acid was added and the tube was placed on ice. The absorbances at 405 nm were read with a Gilford Response spectrophotometer using the samples at time zero for reference blank. The concentration of thrombin generated in the reaction mixture was calculated from the change in absorbance at 405 nm obtained with known active site concentrations of thrombin determined under the same condition as described above. For example, with purified thrombin, the change in absorbance at 405 nm obtained with 100 nM of thrombin was 0.03. This was determined by sampling 25 µL from a mixture

containing 50 uL of 100 nM thrombin and 200 µL of 5 mM neutralized EDTA into 775 µL of 200 µM S-2238 at 37°C and addition of 200 µL of 50% acetic acid at 1 min.

2.14.6. ASSAY OF PROTHROMBIN ACTIVATION IN PURIFIED PROTEIN SYSTEM

Prothrombin activation in a purified protein system was examined at different concentrations of F1.2 in a 2-stage amidolytic assay using chromogenic substrate S-2238. Mixtures of factor Xa with $CaCl_2$, factor Va and Xa with $CaCl_2$, factor Xa with phospholipid and $CaCl_2$, or factor Va, Xa with phospholipid and $CaCl_2$ were incubated at 37°C with F1.2 or Tris-buffered saline containing 0.1 mg/mL of bovine serum albumin. Tris-buffered saline containing 0.1 mg/mL of bovine serum albumin was used as a diluent and the reaction mixtures were made up to 190 µL. After a 3-min incubation, prothrombin activation was started by the addition of 10 µL of 20 µM prothrombin. Then at specific times, 25 µL of reaction mixture was subsampled into a plastic tube containing 30 µL of 5 mM S-2238, 200 µL of 10 mM neutralized EDTA and 545 µL of Tris-buffered saline containing 0.1 mg/mL of bovine serum albumin prewarmed to 37°C for 3 min. At 60 s after the

addition of 25 µL reaction mixture to S-2238 containing EDTA, hydrolysis of S-2238 was stopped by addition of 200 µL of 50% acetic acid; and the absorbance at 405 nm was read in a Gilford Response spectrophotometer. The concentration of thrombin generated in the reaction mixture was calculated from the change in absorbance at 405 nm obtained with known active site concentrations of thrombin determined under the same condition as described above. For example, it was determined that the absorbance at 405 nm obtained with 100 nM of purified thrombin in 1 minute was 0.15. This was determined by adding 25 µL of 100 nM thrombin to a 37°C prewarmed mixture containing 30 µL of 50 mM S-2238, 200 µL of 10 mM neutralized EDTA and 545 µL of Tris-buffered saline with 0.1 mg/mL of bovine serum albumin, and subsequent addition of 200 µL of 50% acetic acid at 60 s.

2.15. IN VIVO ANTITHROMBOTIC EXPERIMENTS

CD1 mice were anesthetized individually by placing them for 2 to 4 min, in a glass container containing cotton balls soaked in diethyl ether. The slightly drowsy mouse was taken out of the container and its tail soaked for 30 s in lukewarm water to expand the tail vein. For each experiment, 0.2 mL of test material was injected intravenously into the tail vein of

the mouse. The concentration of test material was adjusted with 0.15 M NaCl to maintain a total injection volume of 0.2 mL. The end point of each experiment was death of mouse within 5 min.

CHAPTER 3

RESULTS

3.1. INITIAL IDENTIFICATION OF PROTHROMBIN FRAGMENT 1.2 AS AN INHIBITOR OF COAGULATION

In a preliminary experiment (Methods section 2.8.) to isolate an inhibitor of the extrinsic pathway of coagulation, DEAE-cellulose was added to cryo-poor plasma for batch adsorption and elution. Adsorbed and eluted proteins, relatively free of albumin and immunoglobulins when compared with plasma, were selected as the source material for fractionation. Throughout the fractionation scheme, inhibitor activity was monitored by the one-stage prothrombin time assay system (Methods section 2.14.1.), in which the clotting time obtained with pooled normal plasma, tissue factor, and $CaCl_2$ in the presence of fractionated material was compared to that of a buffer control. Since factor VII is one of the constituents in the fraction eluted from DEAE-cellulose the presence of additional factor VII may interfere with the detection of an extrinsic pathway inhibitor. Therefore, factor VII was removed from the DEAE-cellulose fraction by affinity chromatography

with a goat polyclonal anti-human factor VII-Sepharose column. When tested in a prothrombin time assay system with factor VII deficient plasma, the unadsorbed protein fractions had less than 1% factor VII activity. This factor VII-depleted material from the column was found to possess inhibitory activity as it prolonged the prothrombin time of pooled normal plasma by 3 s compared to a buffer control (i.e. 18 s compared to 15 s). Further purification of this inhibitory material was achieved by barium citrate adsorption and elution followed by $(NH_4)_2SO_4$ precipitation. The 40 to 60% $(NH_4)_2SO_4$ precipitated material was then subjected to DEAE-Sepharose chromatography with an increasing NaCl gradient. The elution profile is shown in Fig. 9, and the inhibitory material was found at the latter half of the large protein peak, eluting at 0.25 M NaCl. The fractions containing inhibitory activity was pooled and applied to a Sephacel S-200 column. Results of gel filtration through Sephacel S-200 is shown in Fig. 10. Of the 3 protein peaks obtained, only the last peak, which was homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 11), contained inhibitor activity. A goat was immunized with the inhibitor, and a single immunoprecipitin line was observed in immunoelectrophoresis of the antigen and antibody (Fig. 12). Amino-terminal amino acid sequence of the first 30 residues of

Fig. 9 Ion exchange chromatography on DEAE-Sepharose. The material after $(NH_4)_2SO_4$ precipitation was applied to a column (2.6 x 35 cm) of DEAE-Sepharose. The column was washed with 300 mL 0.05 M Tris-HCl (pH 7.5). Protein was eluted from the column with a linear gradient from 0 to 0.5 M NaCl in 0.05 M Tris-HCl (pH 7.5) over 1500 mL. Fractions (8 mL) were collected at a flow rate of 120 mL/h. Inhibitory material, determined by prolongation of clotting time of normal plasma in a prothrombin time assay system, eluted at the latter half of the large protein peak (fractions 85 to 95; x—x). Absorbance at 280 nm (—). Molar sodium chloride concentration determined by chloride titration (o--o).

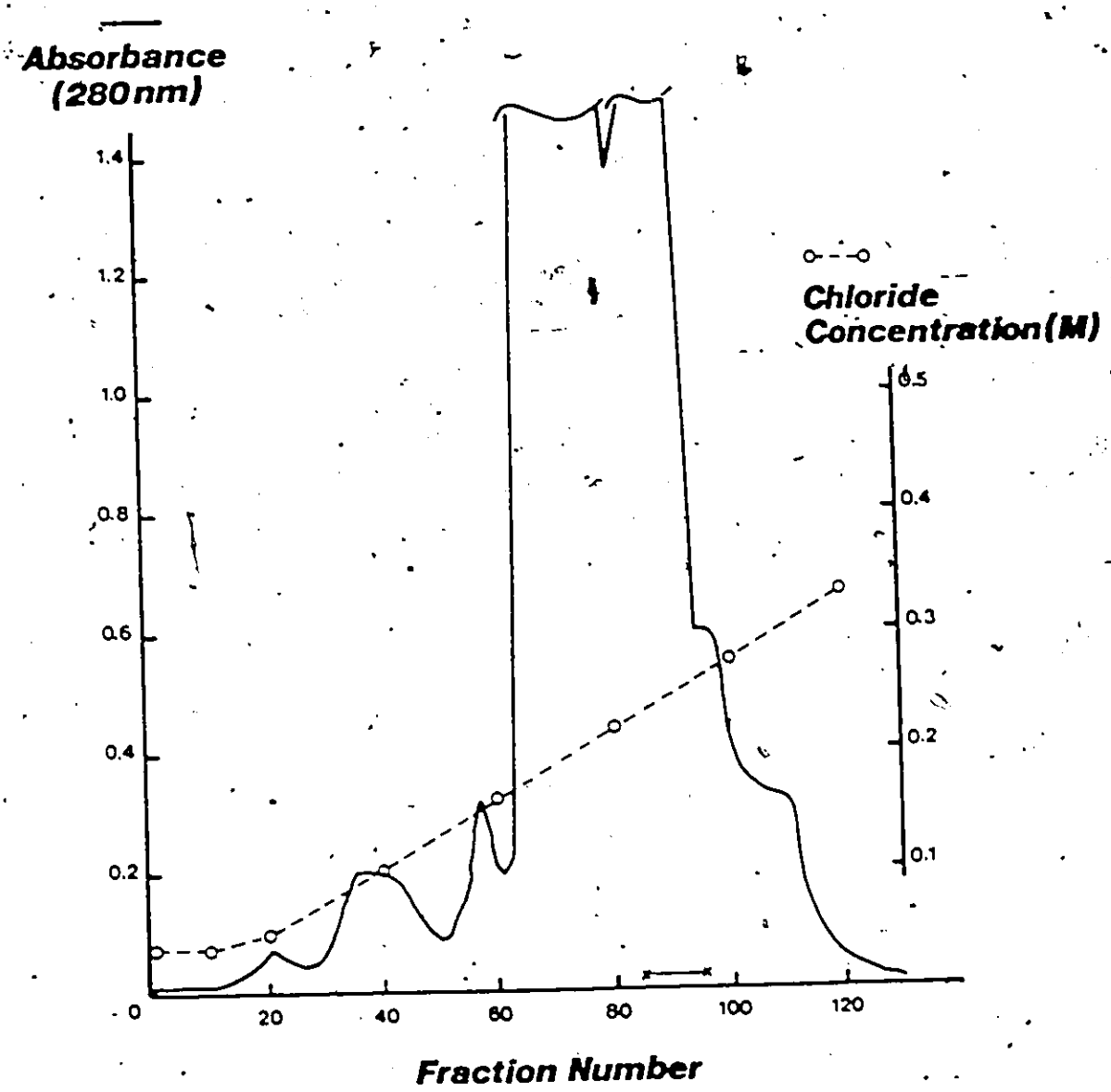

Absorbance
(280nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
Fraction Number
Chloride
Concentration(M)
0.5
0.4
0.3
0.2
0.1

Fig. 10 Gel filtration on Sephacryl S-200. The material after elution from DEAE-Sepharose was concentrated to 6 mL and applied to a column (2.6 x 35 cm) of Sephacryl S-200. Fractions (3 mL) were collected at a flow rate of 20 mL/h. Inhibitory material, determined by prolongation of clotting time of normal plasma in a prothrombin time assay system, eluted at the last protein peak (fractions 28 to 32; x—x). Absorbance at 280 nm (—).

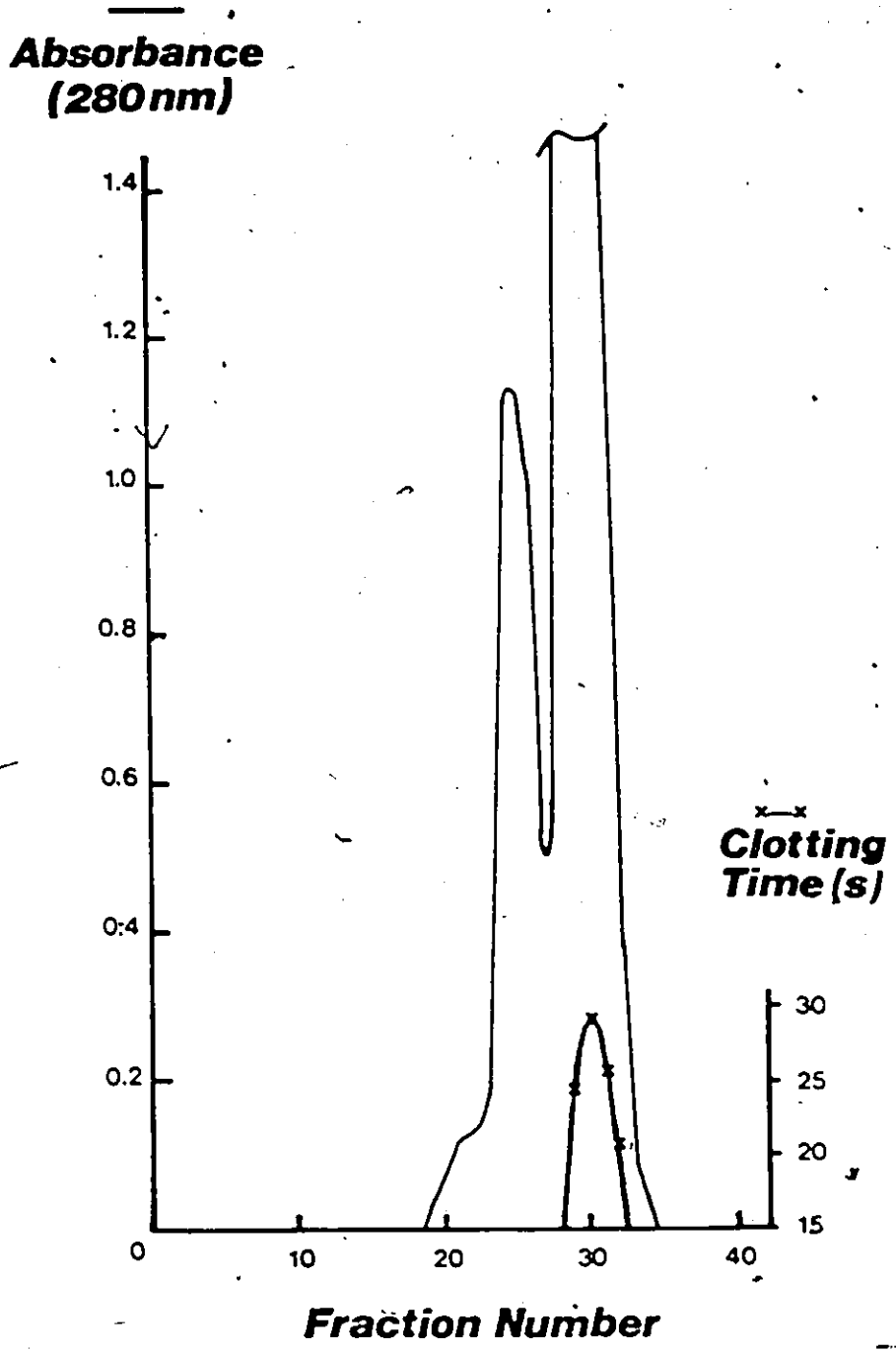

Absorbance (280nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
Clotting Time (s)
30
25
20
15
Fraction Number

Fig. 11 SDS-polyacrylamide gel electrophoresis of initial preparation of prothrombin F1.2 in Tris-buffer system of Laemmli (4% stacking gel, 12% separating gel). Lanes 1 and 2 were molecular weight standards and 30 ug protein respectively before reduction with 2-mercaptoethanol. Lanes 3 and 4 were 30 ug protein and molecular weight standards repectively after reduction with 2-mercaptoethanol. Molecular weight standards were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

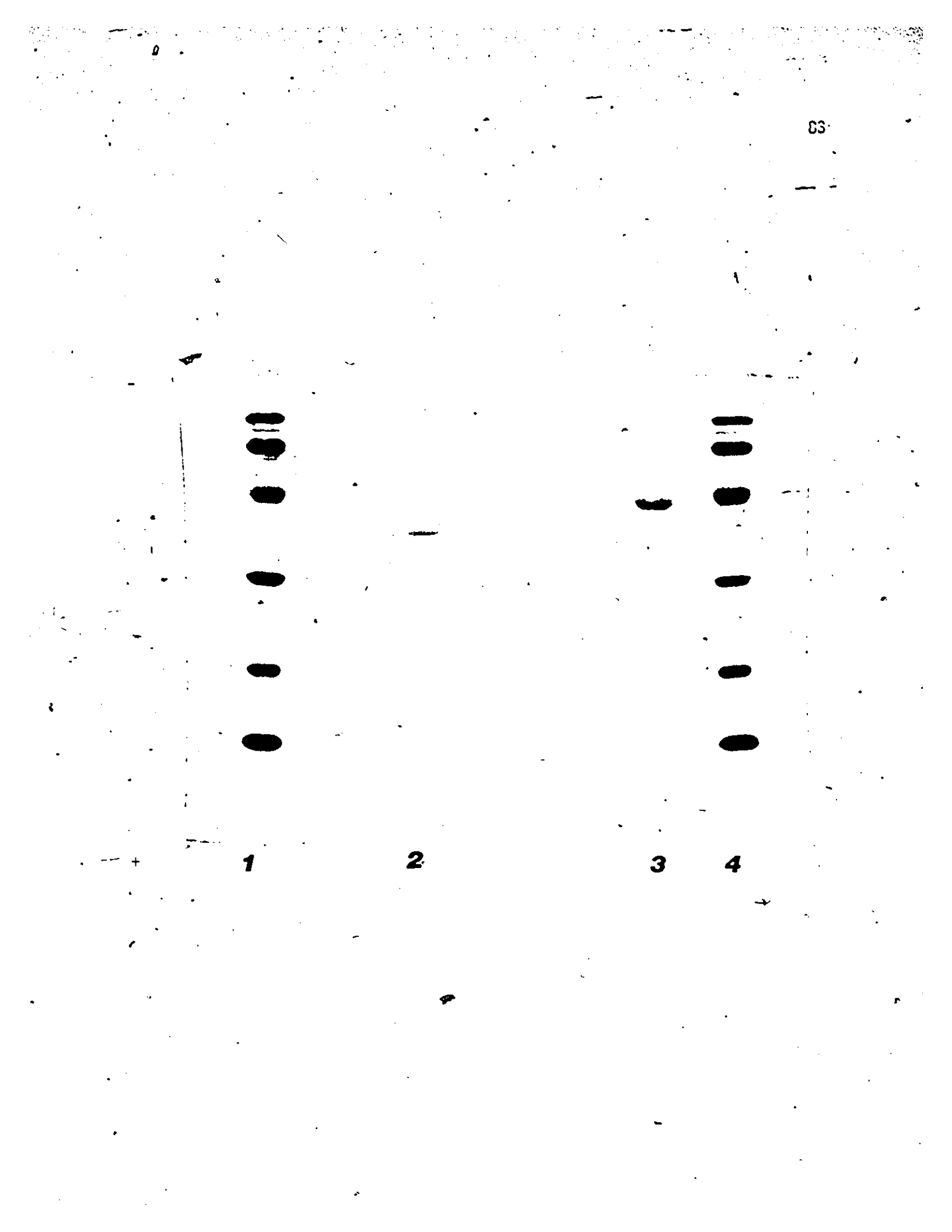

1
2
3
4

Fig. 12 Immunoelectroelectrophoresis of initial preparation of prothrombin F1.2 and goat antibody. Normal human plasma in upper well and F1.2 in lower well.

the inhibitor and the apparent molecular weight on SDS-polyacrylamide gel electrophoresis suggested that the protein was prothrombin F1.2 (Table I). Thus, prothrombin was inadvertently activated during the isolation procedure and preliminary results suggested that prothrombin F1.2 possesses anticoagulant activity.

Table I **Amino-terminal amino acid sequence of purified prothrombin fragment 1.2 in this study.**

1				5						10
Ala	Asn	Thr	Phe	Leu	( )	( )	Val	Arg		Lys
11				15						20
Gly	Asn	Leu	( )	Arg	( )	( )	Val	( )	( )	
21				25						30
Thr	( )	Ser	Tyr	( )	( )	Ala	Phe	( )		Ala

Blank spaces ( ) represent amino acid residues (e.g. gamma-carboxyglutamic acid and cysteine) which could not be identified on the Sequenator. Based on the known sequence of human prothrombin, positions 6, 7, 14, 16, 19, 20, 25, 26, and 29 are gamma-carboxyglutamic acid; and positions 17 and 22 are cysteine (Mann et al., 1981).

3.2. ISOLATION AND CHARACTERIZATION OF PROTHROMBIN FRAGMENT 1.2

Subsequent to the initial experiment (Sections 2.8. and 3.1.), a method for the preparation of prothrombin F1.2 from freshly clotted plasma was developed (Methods section 2.9.).

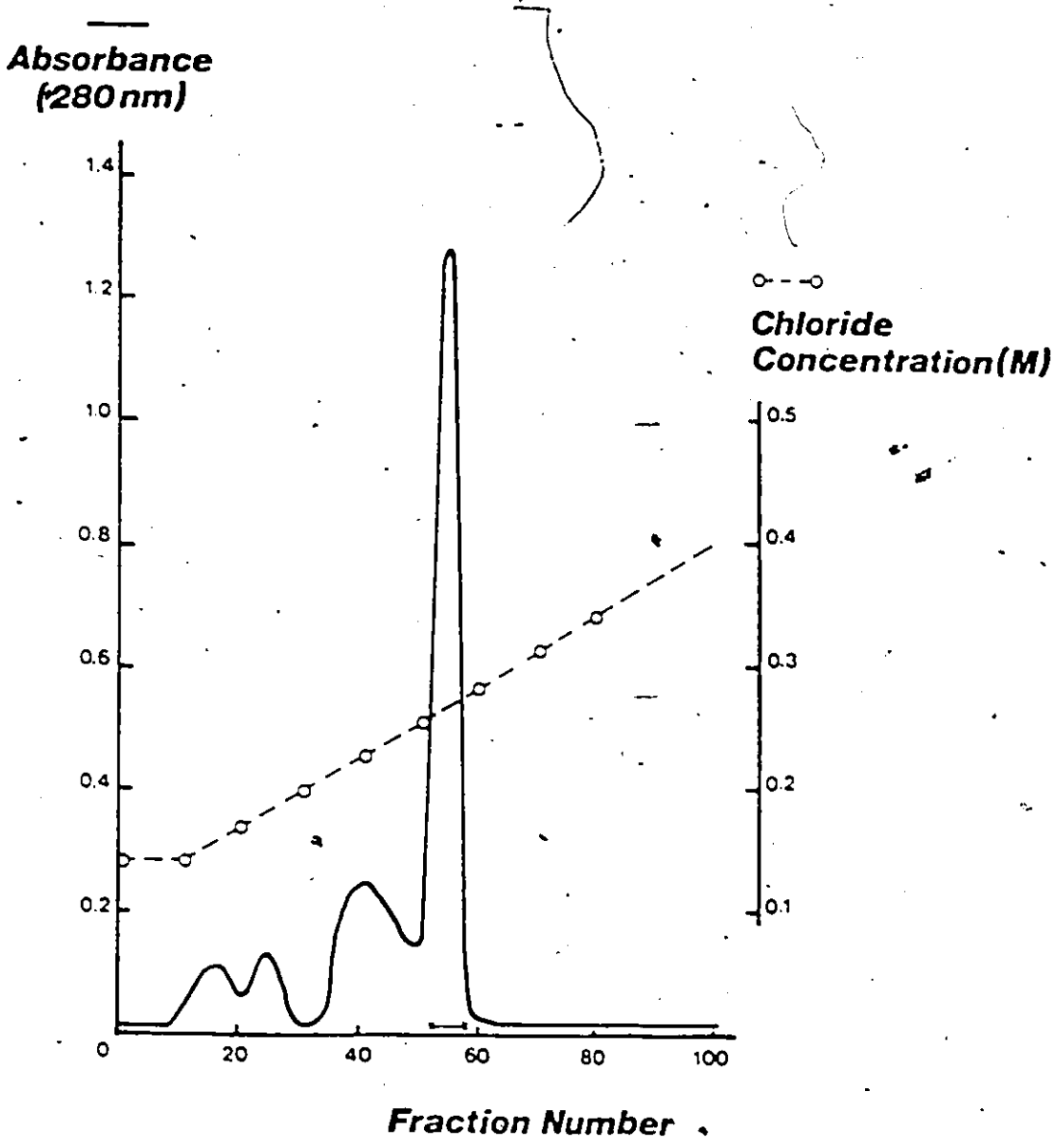
Routinely 25 mg of prothrombin F1.2 was obtained from 4 L of stored plasma. This corresponds to an approximate yield of 7% from starting pooled human plasma (Table II). In this purification procedure, the plasma was clotted by recalcification. Heparin was added to the serum before fractionation to enhance inactivation of thrombin and prevent degradation of F1.2 to F1 and F2. Inclusion of benzamidine in the early stages of the fractionation scheme further prevented occurrence of fragment 1 and fragment 2 as contaminants in the final product. Subsequent barium citrate adsorption and elution followed by 30 to 60% $(NH_4)_2SO_4$ precipitation was an effective means of separating vitamin K-dependent coagulation factors and F1.2 from other proteins in serum. The elution profile of DEAE-Sepharose column chromatography is shown in Fig. 13. F1.2 eluted as the major protein peak from the DEAE-Sepharose column and represented approximately 95% of the

Table II Recovery of prothrombin F1.2.

	mg/L
Prothrombin concentration in starting plasma	178
Theoretical F1.2 concentration (assuming prothrombin conversion to F1.2 was 100% and that F1.2 represents half of the prothrombin molecule)	89

Recovery = 6.25/89 x 100% = 7%

Fig. 13 Elution pattern of prothrombin F1.2 from DEAE-Sepharose. The material after $(NH_4)_2SO_4$ precipitation was applied to a column (2.6 x 35 cm) of DEAE-Sepharose. The column was washed with 300 mL 0.1 M NaCl in 0.05 M Tris-HCl at pH 7.5 containing 0.02 M benzamidine. Protein was eluted from the column with a linear gradient from 0.1 to 0.5 M NaCl in 0.05 M Tris-HCl at pH 7.5 containing 0.02 M benzamidine over 1.0 L. Fractions (8 mL) were collected at a flow rate of 120 mL/h. Prothrombin F1.2, determined by double diffusion with antibody developed against F1.2, eluted under the largest protein peak (fractions 52 to 58; x—x). A_{280} was determined on samples diluted 10-fold from which the A_{280} of a 10-fold dilution of the elution buffer was subtracted. Absorbance at 280 nm (—). Molar NaCl concentration determined by chloride titration (o--o)


Absorbance
(280nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
Chloride
Concentration(M)
0.5
0.4
0.3
0.2
0.1
Fraction Number

protein in that fraction as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Fig. 14). The remaining contaminants, activated coagulation factors and higher molecular weight material, were removed by benzamidine-Sepharose and Cibacron Blue-agarose column chromatography respectively.

The purified preparation of F1.2 was without detectable amidolytic activity on the following chromogenic substrates: S-2222, S-2238, S-2251, and S-2302, upon testing at a protein concentration of 1.0 g/L for 24 h at 37°C. In addition, F1.2 did not inhibit amidolytic activities of thrombin and factor Xa on their respective chromogenic substrates (Table III). The purified preparation of F1.2 was homogeneous by ultracentrifugation (Fig. 15) and electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (Fig. 16). The sedimentation coefficient was 2.15 S. A single polypeptide chain, as determined by SDS-polyacrylamide gel electrophoresis in the absence and presence of 2-mercaptoethanol, with an apparent molecular weight of 36,000 was observed for human prothrombin F1.2. A single immunoprecipitin line was observed in immunoelectrophoresis of the purified F1.2 and a goat antibody produced with F1.2, prepared with Methods section 2.8., as the immunogen (Fig. 17).

Fig. 14 SDS-polyacrylamide gel electrophoresis of fractions 52 to 58 from DEAE-Sepharose column in Tris-buffer system of Laemmli (4% stacking gel, 12% separating gel). Lanes 1 and 2 were molecular weight standards and 30 µg protein, respectively. Molecular weight standards were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

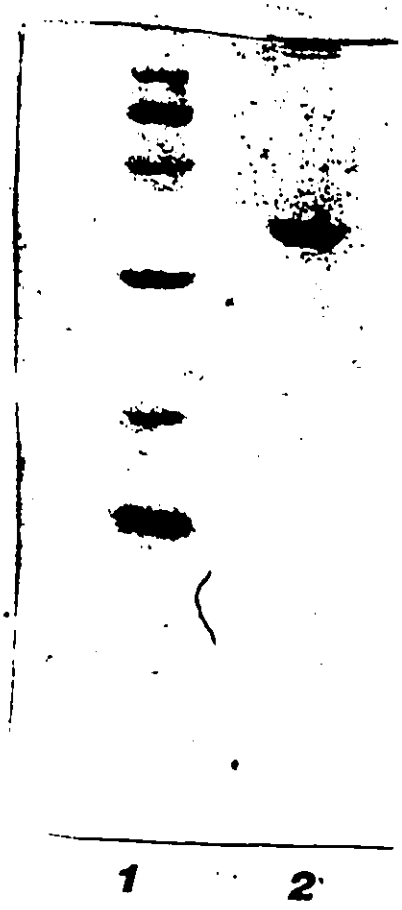

1
2

Table III

A. Assessment of amidolytic activity of fragment 1.2.

Absorbance (single determination) at 405 nm after 24 h incubation at 37°C (for details, see Methods section 2.9.).

	Fl.2	Xa	thrombin	plasmin	kallikrein
S-2222	0	>2.0	-	-	-
S-2238	0	-	>2.0	-	-
S-2251	0	-	-	>2.0	-
S-2303	0	-	-	-	>2.0

B. Assessment of effects of fragment 1.2 on amidolytic activities of factor Xa and thrombin on their respective chromogenic substrates.

Absorbance (mean of 2 determinations) for 1-min incubation of reaction mixture at 37°C (for details, see Methods section 2.9.).

	Fl.2	Buffer
Factor Xa + S-2222	0.26	0.25
Thrombin + S-2238	0.52	0.54

Fig. 15 Ultracentrifugation analysis of prothrombin F1.2.

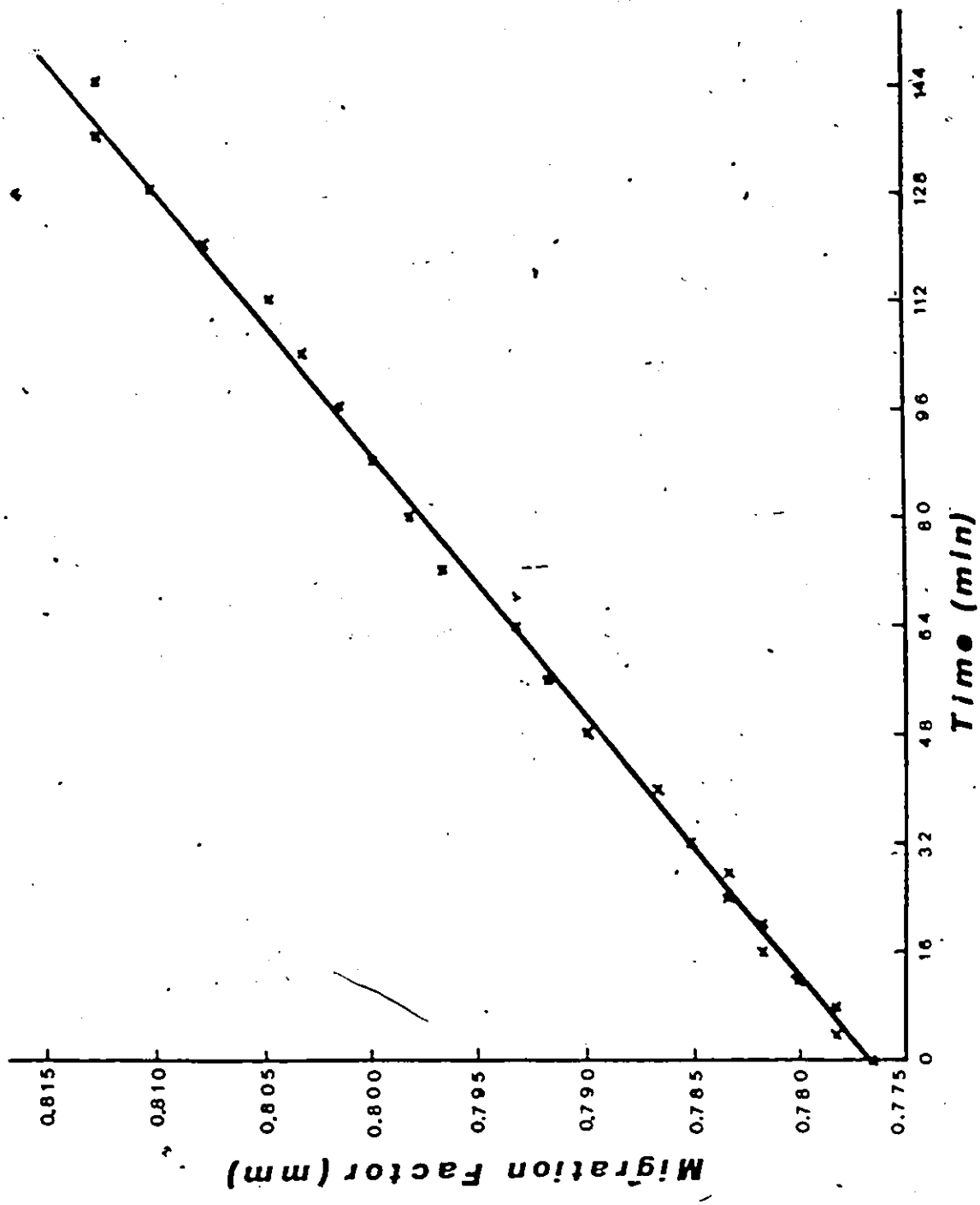

Migration Factor (mm)
0.815
0.810
0.805
0.800
0.795
0.790
0.785
0.780
0.775
Time (min)
0
16
32
48
64
80
96
112
128
144

Fig. 16 SDS-polyacrylamide gel electrophoresis of final preparation of prothrombin F1.2 in Tris-buffer system of Laemmli (4% stacking gel, 12% separating gel). Lanes 1,2,3 were molecular weight standards, 10 µg protein, and 30 µg protein respectively before reduction with 2-mercaptoethanol. Lanes 4,5,6 were 10 µg protein, 30 µg protein, molecular weight standards respectively after reduction with 2-mercaptoethanol. Molecular weight standards were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

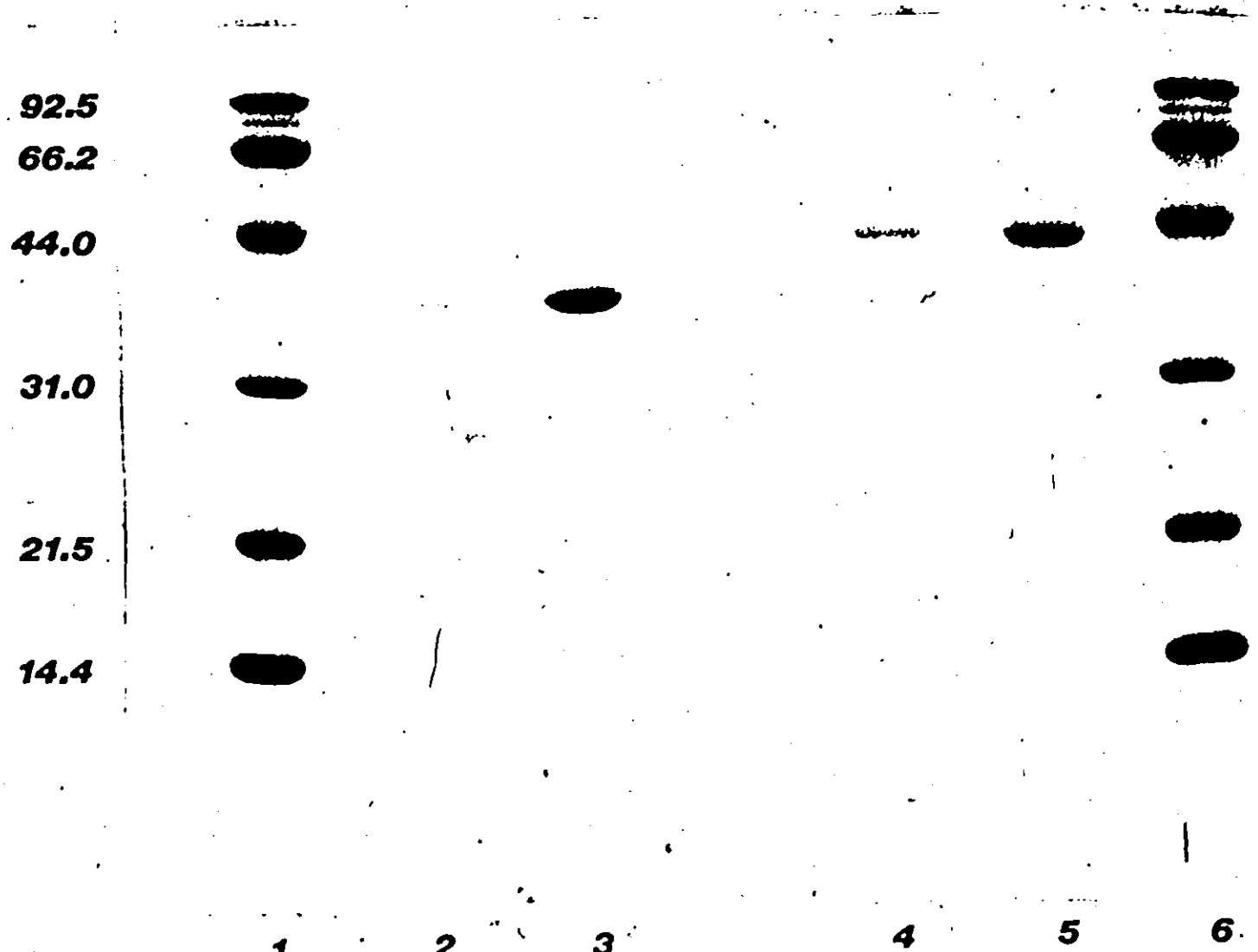

92.5
66.2
44.0
31.0
21.5
14.4
1
2
3
4
5
6

Fig. 17 Immunoelectrophoresis of prothrombin F1.2 and antibody to prothrombin F1.2.

The amino acid composition of F1.2 from a single determination is shown in Table IV. The extinction coefficient $E_{280}^{1\%}$ of F1.2 determined from amino acid composition was 11.7. The amino-terminal amino acid sequence for the first 30 residues of F1.2 is identical to that shown in Table I.

Table IV Comparison of amino acid composition of human prothrombin fragment 1.2.

Amino acid	Residues/mole This study	Esmon (1974)	Aronson (1977)
Asp	28	32.4	27.1
Thr	30	15.0	20.4
Ser	17	22.8	15.3
Glu	36	37.8	40.0
Pro	15	19.6	13.7
Gly	19	24.1	18.4
Ala	23	20.0	21.2
Cys	4	13.3	18.4
Val	14	14.5	14.5
Met	4	0.8	1.5
Ile	9	4.9	5.3
Leu	14	19.2	17.5
Tyr	9	7.3	13.1
Phe	12	7.0	7.5
Lys	10	7.1	5.2
His	5	2.2	8.2
Arg	21	22.5	16.2
	271	270.5	263.5

3.3. ANTICOAGULANT PROPERTIES OF FRAGMENT 1.2 IN VITRO

3.3.1. EFFECT OF PROTHROMBIN FRAGMENT 1.2 ON PROTHROMBIN TIME IN NORMAL PLASMA

The in vitro anticoagulant effect of fragment 1.2 was initially investigated by performing prothrombin time in reaction mixtures containing normal plasma, $CaCl_2$, tissue factor and varying concentrations of exogenous prothrombin F1.2 added to the system. Fig. 18 illustrates the dose response curve for different concentrations of exogenous F1.2 in a reaction mixture containing constant amounts of normal plasma, Ca^{2+} and tissue factor. A linear relationship was obtained between the clotting time and concentration of exogenous F1.2. By linear regression analysis, it was determined that each μM of exogenous fragment added to the system could prolong the prothrombin system by 0.9 s.

Possible modes of anticoagulant action of F1.2 were next examined by varying the concentration of tissue factor while maintaining constant amounts of normal plasma and $CaCl_2$ in the reaction mixture. Fig. 19 summarizes data obtained in these experiments. The standard dose response curve for

Fig. 18 Plot of clotting time against concentration of exogenous F1.2 in a prothrombin time assay system composed of 75 µL pooled normal plasma, 75 µL different dilutions of F1.2 in Tris-buffered saline and 150 µL tissue factor (Thromborel S). Each data point is the mean of 3 determinations. The standard error of the mean was less than 10%.

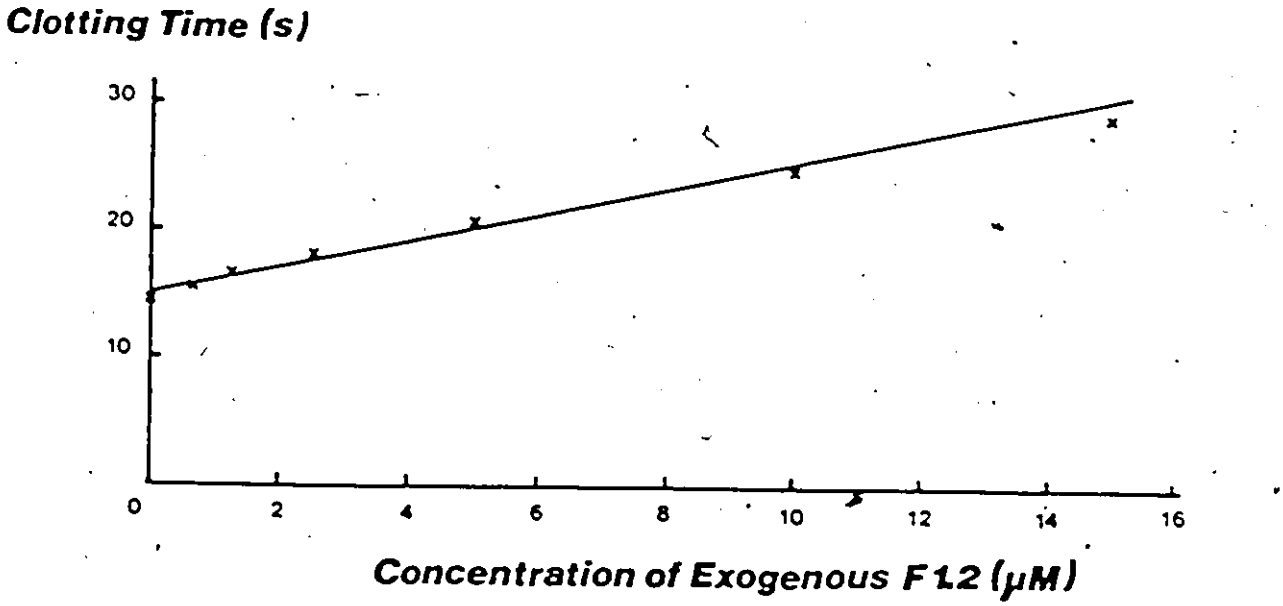
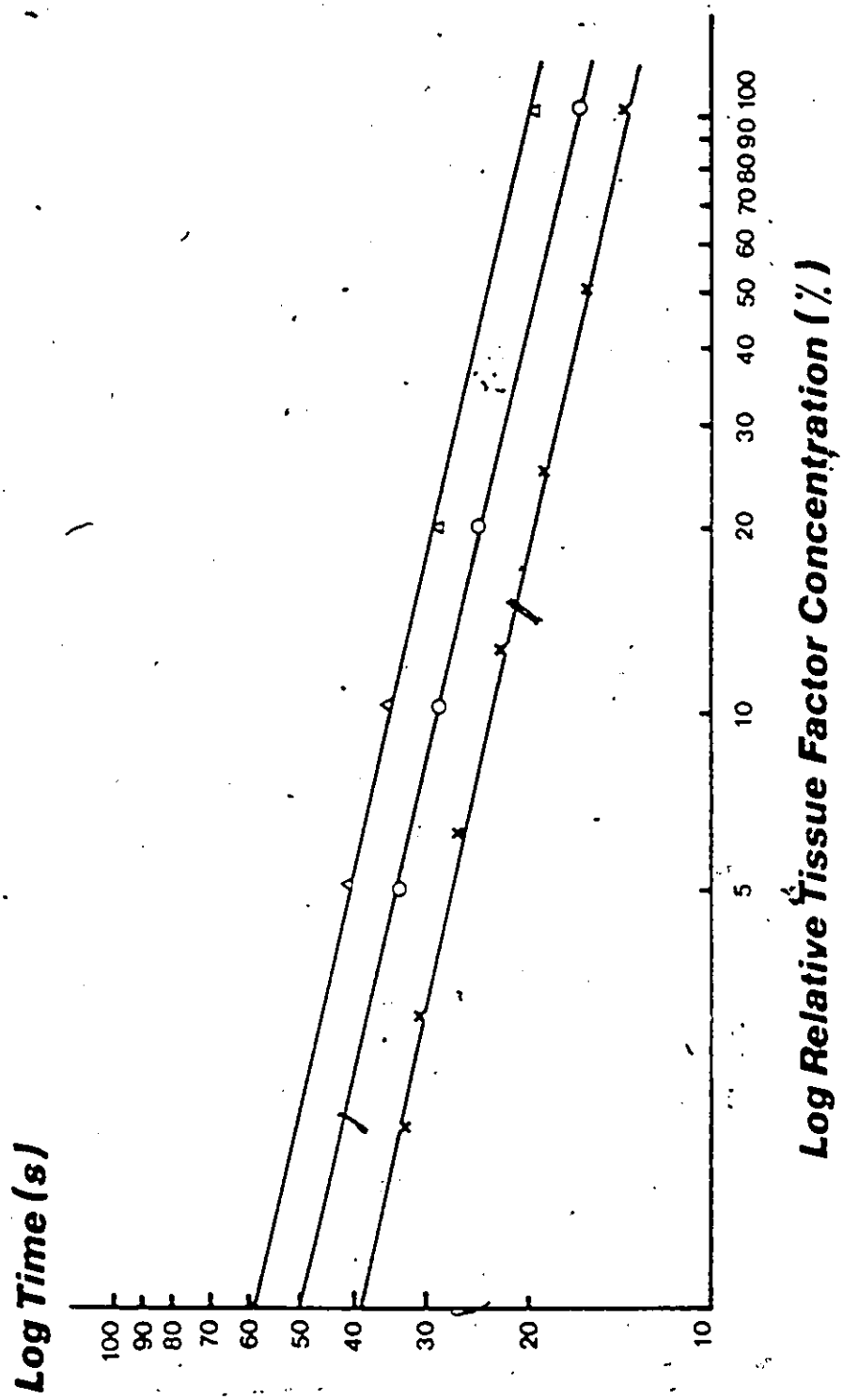

Clotting Time (s)
30
20
10
0
2
4
6
8
10
12
14
16
Concentration of Exogenous F1.2 (μM)

Fig. 19 Log-log plot of prothrombin time against dilution of tissue factor (Thromborel S) in a prothrombin time assay system composed of 75 μL pooled normal plasma, 75 μL of F1.2 and 150 μL of tissue factor. F1.2 was diluted in Tris-buffered saline and tissue factor was diluted in 12.5 mM $CaCl_2$. Prothrombin time obtained with no exogenous F1.2 (x—x); with 2 μM exogenous F1.2 (o-o); and with 5 μM exogenous F1.2 (△—△). Each data point is the mean of 2 determinations.


Log Time (s)
100
90
80
70
60
50
40
30
20
10
5
10
20
30
40
50
60
70
80
90
100
Log Relative Tissue Factor Concentration (%)

clotting time against different concentrations of tissue factor was linear on log-log transformation, and this relationship was proportionately maintained in the presence of exogenous F1.2, i.e., similar slopes were obtained with different F1.2 concentrations. Thus, the concentrations of tissue factor did not appear to alter the kinetics of anticoagulant action of F1.2 in the prothrombin time assay system, suggesting that the anticoagulant activity was not directed against tissue factor. It was further observed that incubating F1.2 with tissue factor in the presence of $CaCl_2$ for 0 to 30 min at 37°C before the addition of normal plasma did not alter the clotting time obtained.

In another experiment, F1.2 was incubated with the following: (i) particulate tissue factor and $CaCl_2$, (ii) particulate tissue factor, $CaCl_2$ and factor VIIa, (iii) particulate tissue factor, $CaCl_2$, factors VIIa and Xa. As a control experiment, dialyzed defibrinated plasma was incubated with particulate tissue factor and $CaCl_2$, because Hubbard and Jennings (1986) reported that dialyzed defibrinated plasma could inhibit tissue factor-factor VIIa activity. After 60 min incubation, the tissue factor pellet was isolated, washed and assayed for tissue factor activity. The results shown in Table V indicate that F1.2 by itself did not diminish tissue factor

Table V **Clotting times obtained with resuspended tissue factor pellets after preincubation with prothrombin fragment 1.2 or buffer in the presence or absence of factor VIIa or factors VIIa and Xa.**

Each value is the mean of 3 determinations (for details, see Methods section 2.14.3.).

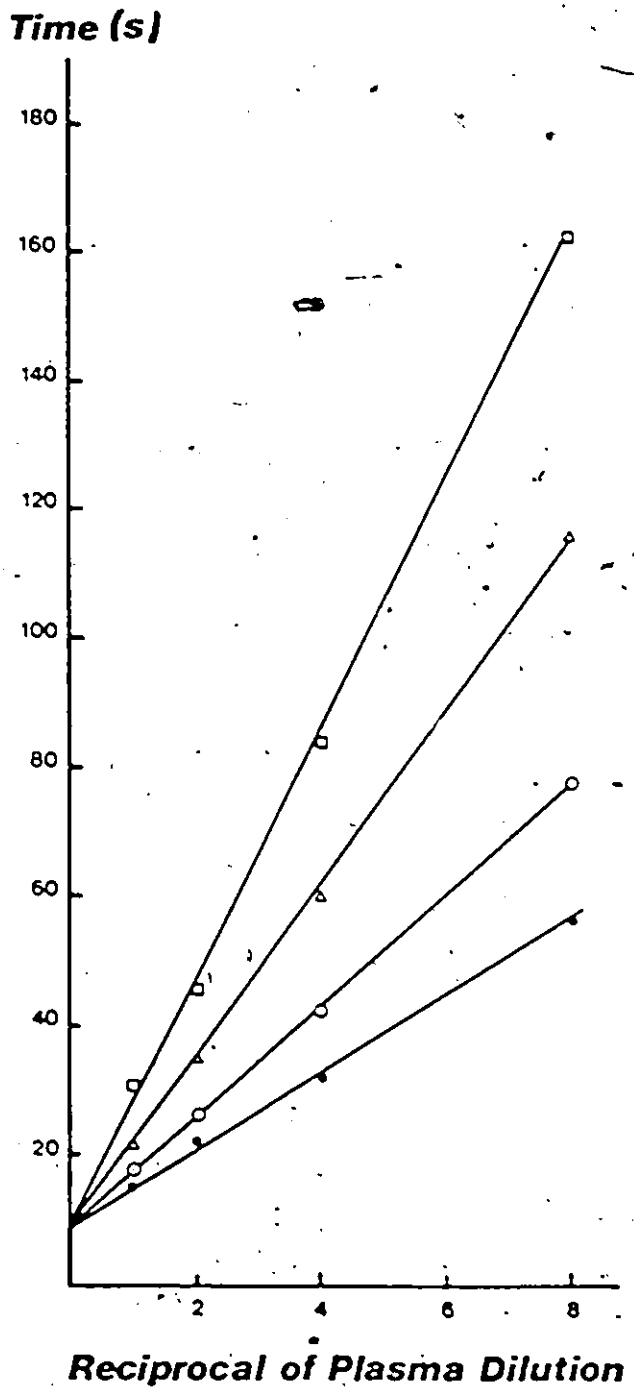
	F 1.2	Buffer
TF + $CaCl_2$	23.4 ± 0.7 s	23.4 ± 0.6 s
TF + $CaCl_2$ + VIIa	20.5 ± 0.6 s	19.7 ± 0.5 s
TF + $CaCl_2$ + VIIa + Xa	23.9 ± 0.7 s	23.5 ± 0.8 s

In a control experiment, the clotting time of TF + $CaCl_2$ + dialyzed defibrinated plasma was 34.0 ± 0.8 s.

activity in the presence or absence of factors VIIa and Xa. In contrast, inhibition of tissue factor-activity was obtained by incubation of dialyzed defibrinated plasma with particulate tissue factor.

The anticoagulant action of F1.2 was then investigated in a reaction system composed of different concentrations of normal plasma but constant amounts of tissue factor and $CaCl_2$. The results are shown in Fig. 20, with clotting time plotted against reciprocal of plasma dilution. It has been proposed by Duncan and Lloyd (1978) that these plots can be thought of as Lineweaver-Burk plots and that the prothrombin time is proportional to the reciprocal of the reaction velocity and reciprocal of plasma dilution is proportional to the reciprocal of the substrate concentration. Although the one-stage prothrombin time is complex with several reactants, these plots approximate a straight line over the 8-fold dilution range of the present experiments. Since the lines obtained for the differrent concentrations of F1.2 mutually intersect on the ordinate, the pattern fits that described for competitive inhibition, i.e., the slope is affected by the presence of inhibitor but the intercepts remain constant. Thus, the results suggest that F1.2 can compete with the substrate (prothrombin) for the free enzyme (prothrombinase complex); and

Fig. 20 Prothombin time plotted against plasma dilution for 3 concentrations of exogenous F1.2 : no exogenous F1.2 (•—•); 1.5 μM exogenous F1.2 (o—o); 5 μM exogenous F1.2 (△—△); 10 μM exogenous F1.2 (□—□). F1.2 and pooled normal plasma were diluted in Tris-buffered saline, and the assay system was maintained at 75 μL plasma, 75 μL F1.2 and 150 μL tissue factor (Thromborel S). Each data point is the mean of 3 determinations. The standard error of the mean was less than 10%.


Time (s)
180
160
140
120
100
80
60
40
20
2
4
6
8
Reciprocal of Plasma Dilution

prolongation of clotting time may be related to delay in formation of threshold quantities of thrombin required for clotting.

3.3.2. EFFECT OF PROTHROMBIN FRAGMENT 1.2 ON ACTIVATED PARTIAL THROMBOPLASTIN TIME IN NORMAL PLASMA

The effect of F1.2 on intrinsic pathway of coagulation was assessed by performing activated partial thromboplastin time in reaction mixtures containing normal plasma, activated partial thromboplastin reagent, $CaCl_2$ and different concentrations of exogenous F1.2 added to the reaction system. As shown in Fig. 21, a linear dose response relationship was obtained between clotting time and concentration of exogenous F1.2. By linear regression analysis, it was determined that each μM of exogenous F1.2 added to the test system could prolong the activated partial thromboplastin time by 2.4 s.

The anticoagulant action of F1.2 *in vitro* was investigated further by varying the concentration of normal plasma while maintaining constant amounts of activated partial thromboplastin reagent and $CaCl_2$. The plots of clotting time versus reciprocal of plasma dilution shows that the slope is affected by the presence of F1.2 but the intercepts remain constant (Fig. 22). Adopting the approach of Duncan and Lloyd (1978) for analysis of results, a competitive inhibition pattern is obtained in the activated partial thromboplastin

Fig. 21 Plot of clotting time against concentration of exogenous F1.2 in an activated partial thromboplastin time assay system composed of 0.1 mL pooled normal plasma, 0.1 mL different dilutions of F1.2 in Tris-buffered saline, 0.1 mL activated partial thromboplastin reagent and 0.1 mL 25 mM $CaCl_2$. Each data point is the mean of 3 determinations. The standard error of the mean was less than 10%.

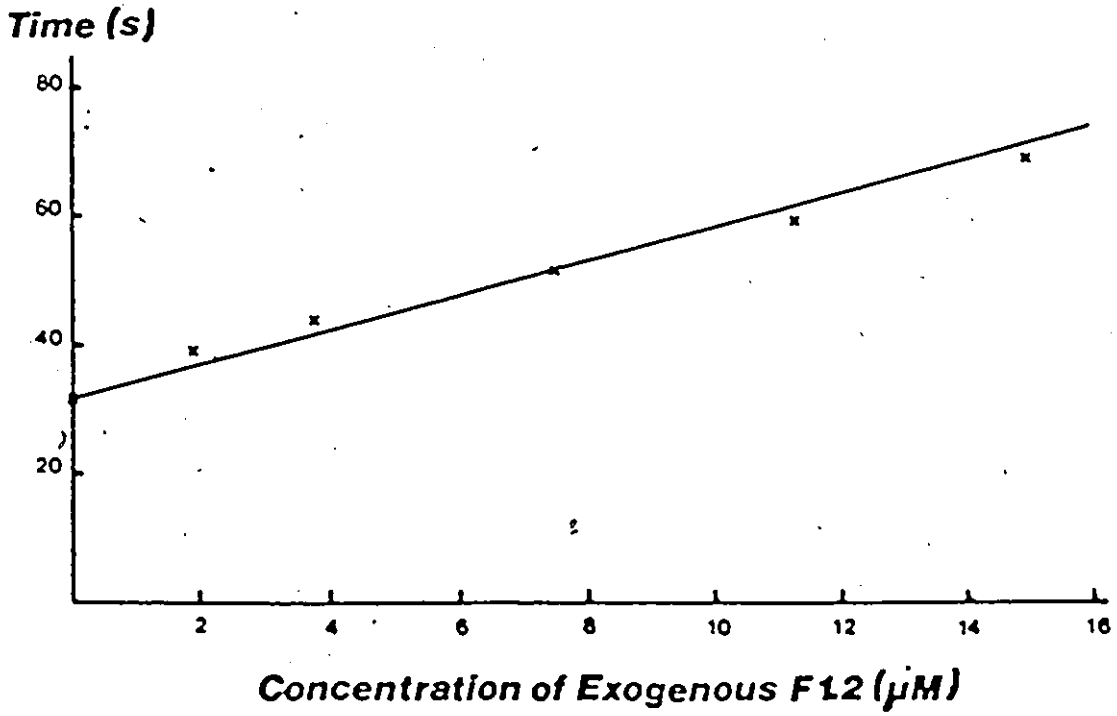
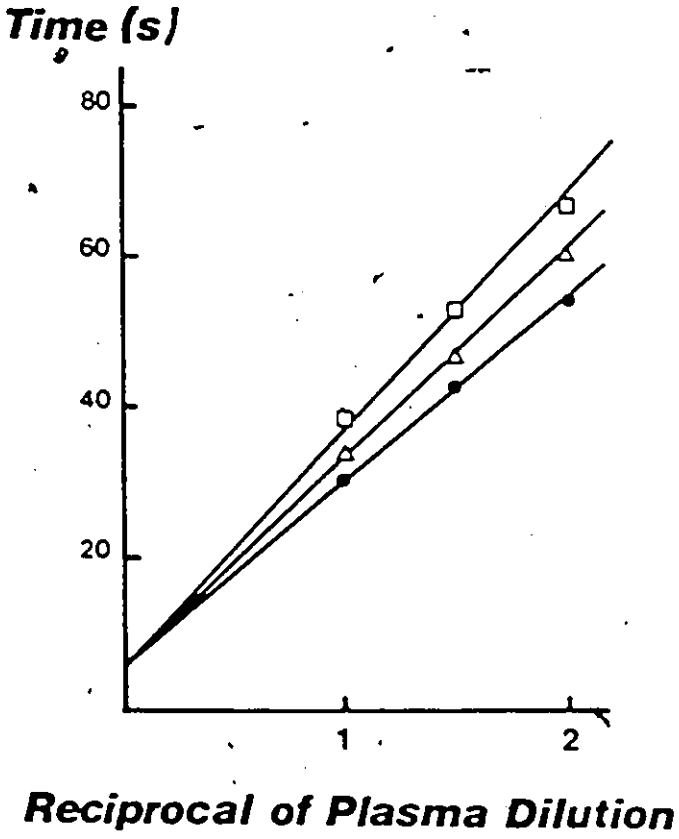

Time (s)
80
60
40
20
2
4
6
8
10
12
14
16
Concentration of Exogenous F12 (μM)

Fig. 22 Activated partial thromboplastin time plotted against plasma dilution for 2 concentrations of exogenous F1.2 : no exogenous F1.2 (●—●); 1.5 μM exogenous F1.2 (△—△); 3 μM exogenous F1.2 (□—□). F1.2 and pooled normal plasma were diluted in Tris-buffered saline, and the assay system was maintained at 0.1 mL plasma, 0.1 mL F1.2, 0.1 mL activated partial thromboplastin reagent and 0.1 mL 25 mM $CaCl_2$. Each data point is the mean of 3 determinations. The standard error of the mean was less than 10%.


Time (s)
80
60
40
20
1
2
Reciprocal of Plasma Dilution

system. This observation is consistent with the hypothesis that F1.2 competes with the substrate (prothrombin) for the free enzyme (prothrombinase complex).

3.3.3. EFFECT OF PROTHROMBIN FRAGMENT 1.2 ON THROMBIN GENERATION IN DEFIBRINATED PLASMA

Since it was observed that F1.2, without inhibiting the activities of factor Xa and thrombin (Results section 3.2.), could prolong both prothrombin time and activated partial thromboplastin time in whole plasma (Results sections 3.3.1. and 3.3.2.), the effect of F1.2 on thrombin generation was then studied in a defibrinated plasma system. Figs. 23 and 24 quantify the amidolytic activity of thrombin observed when tissue factor and $CaCl_2$ or activated partial thromboplastin reagent and $CaCl_2$ were used to initiate thrombin formation. In the control defibrinated plasma system, generally an initial rapid prothrombin activation phase is followed by thrombin inactivation phase. The presence of exogenous F1.2 delayed the appearance of thrombin activity in both extrinsic and intrinsic pathway systems. In the extrinsic pathway, in the absence of F1.2, maximal thrombin activity was observed at about 15 s. The presence of F1.2 delayed the appearance of similar maximal

Fig. 23 Effect of exogenous F1.2 on thrombin generation initiated with tissue factor (Thromborel S) in a defibrinated plasma system. With no exogenous F1.2 (x—x); with 5 μM exogenous F1.2 (o—o); with 10 μM exogenous F1.2 (Δ—Δ); with 20 μM exogenous F1.2 (□—□). For details, see Methods section 2.14.5. Each data point is the mean of 2 determinations.

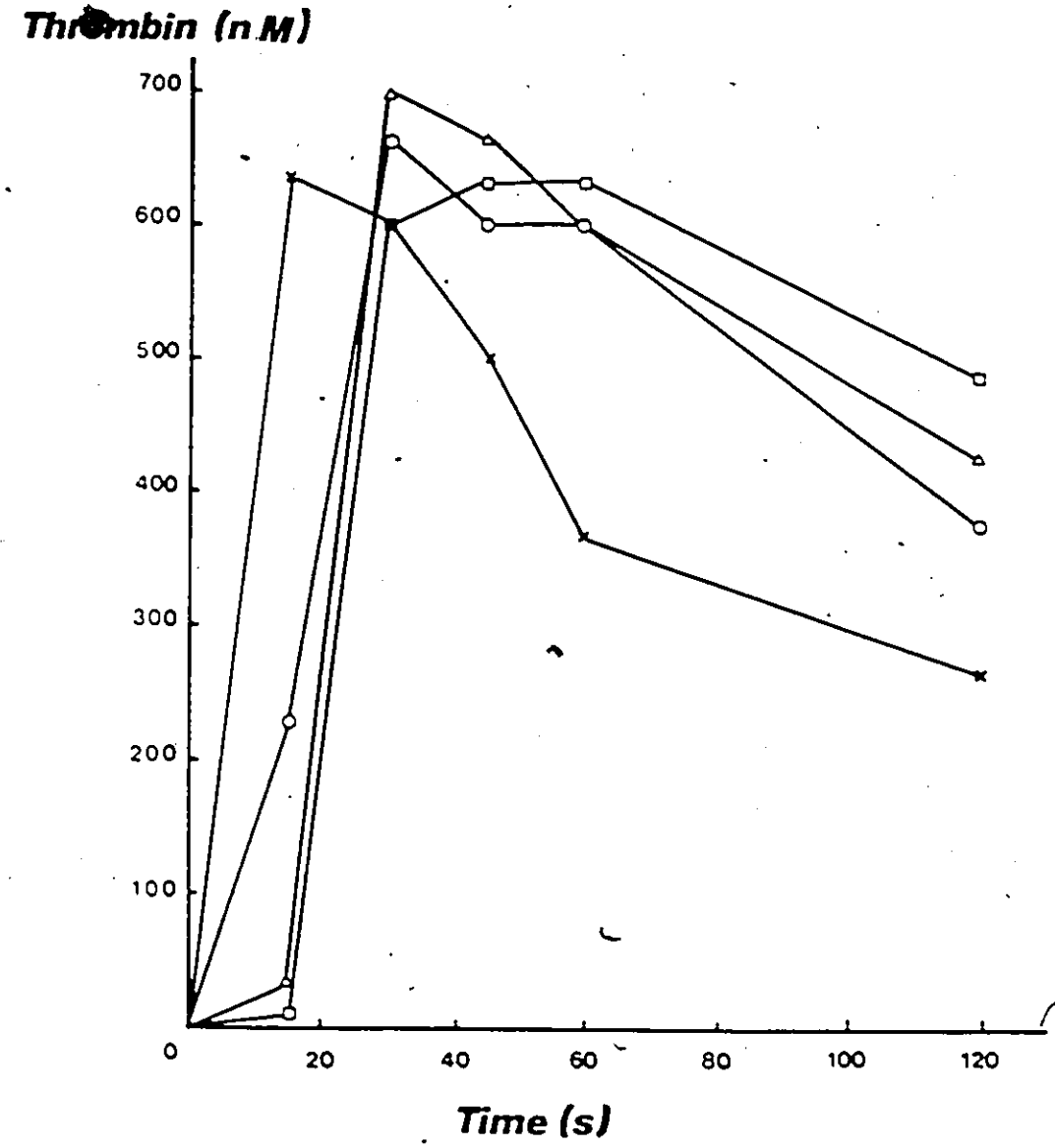
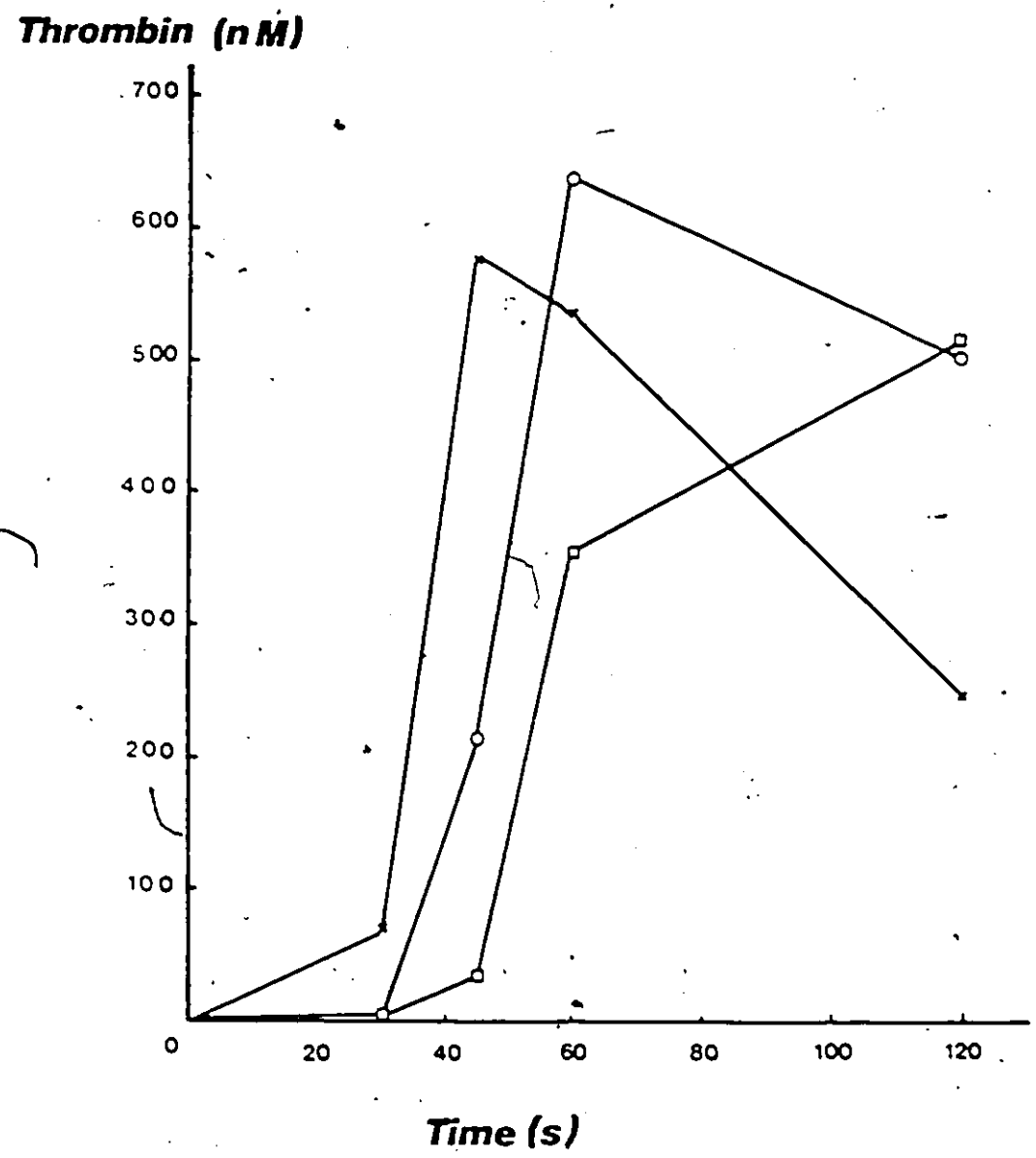

Thrombin (n M)
700
600
500
400
300
200
100
0
20
40
60
80
100
120
Time (s)

Fig. 24 Effect of exogenous $F1_{,}2$ on thrombin generation initiated with activated partial thromboplastin reagent in a defibrinated plasma system. With no exogenous F1.2 (x—x); with 5 μM exogenous F1.2 (o—o); with 10-μM exogenous F1.2 (□—□). For details, see Methods section 2.14.5. Each data point is the mean of 2 determinations.


Thrombin (nM)
700
600
500
400
300
200
100
0
20
40
60
80
100
120
Time (s)

thrombin activity to 30 s. In the intrinsic pathway, maximal thrombin activity was shifted from 45 s to at least 60 s. These experiments provided indirect evidence that the mode of action of exogenous F1.2 on prolongation of prothrombin time and activated partial thromboplastin time in whole plasma was caused by a delay in formation of threshold quantities of thrombin required for clotting, irrespective of the trigger for initiating prothrombin activation. While these results are consistent with the delay of thrombin formation, the site of action of F1.2 is not known.

3.3.4. EFFECT OF PROTHROMBIN FRAGMENT 1.2 ON FACTOR X ACTIVATION

To explore sites of action of F1.2 as an inhibitor of prothrombin activation, the effect of F1.2 on factor X activation was assessed in both defibrinated plasma system and purified protein system (Methods section 2.14.4.). Reaction mixtures contained defibrinated plasma, or purified factor X, purified factor VIIa and tissue factor-$CaCl_2$ in the presence or absence of F1.2. Factor Xa activity was assayed by its activity on chromogenic substrate S-2222. As can be seen from Table VI, the activation of factor X was not inhibited by 10 μM fragment 1.2. Therefore, the delay in thrombin generation, associated with the presence of F1.2 as observed in previous experiments, was not related to decreased activation of factor X.

Table VI **Factor X activation as assayed by amidolytic assay with S-2222** (for details, see Methods section 2.14.4.).

A. In defibrinated plasma system, factor Xa concentration in nM (each value is the mean of 2 determinations).

Incubation time	F1.2	Buffer
3 min	1.0	1.0
5 min	1.0	1.0

B. In purified protein system with purified factors VIIa and X, factor Xa concentration in nM (each value is the mean of 2 determinations).

Incubation time	F1.2	Buffer
5 min	21.9	21.5
10 min	28.1	27.9

3.3.5. EFFECT OF PROTHROMBIN FRAGMENT 1.2 ON THROMBIN GENERATION IN PURIFIED PROTEIN SYSTEM

The results of the previous experiments indicated that the anticoagulant activity of F1.2 was related to its presence during the conversion of prothrombin to thrombin. Consequently, the effect of F1.2 on various components of the prothrombinase complex was studied in purified protein systems to further define the mechanism of inhibition of F1.2 on coagulation (Methods section 2.14.6.). The results of these experiments are summarized in Fig. 25 to 28. F1.2 did not inhibit activation of prothrombin by factor Xa and $CaCl_2$ or factors Va, Xa and $CaCl_2$. However, F1.2 inhibited thrombin generation when reaction mixtures contained coagulant phospholipids. Compared to the buffer control, 2 μM F1.2 inhibited thrombin generation by 87% at 15 min in a reaction mixture containing phospholipid, factor Xa and $CaCl_2$. At 2 μM, F1.2 inhibited thrombin generation by only 30% at 1 min in a reaction mixture containing factor Va, phospholipid, factor Xa and $CaCl_2$. The results show that F1.2 inhibits prothrombin activation and exerts its anticoagulant activity by competing with prothrombin for phospholipid binding sites. The presence

Fig. 25 Effect of F1.2 on thrombin generation in a purified protein system composed of 90 nM factor Xa, 10 mM $CaCl_2$ and 1 μM prothrombin. With no F1.2 (x—x); with 4 μM F1.2 (•—•). For details, see Methods section 2.14.6. Each data point is the mean of 2 determinations.

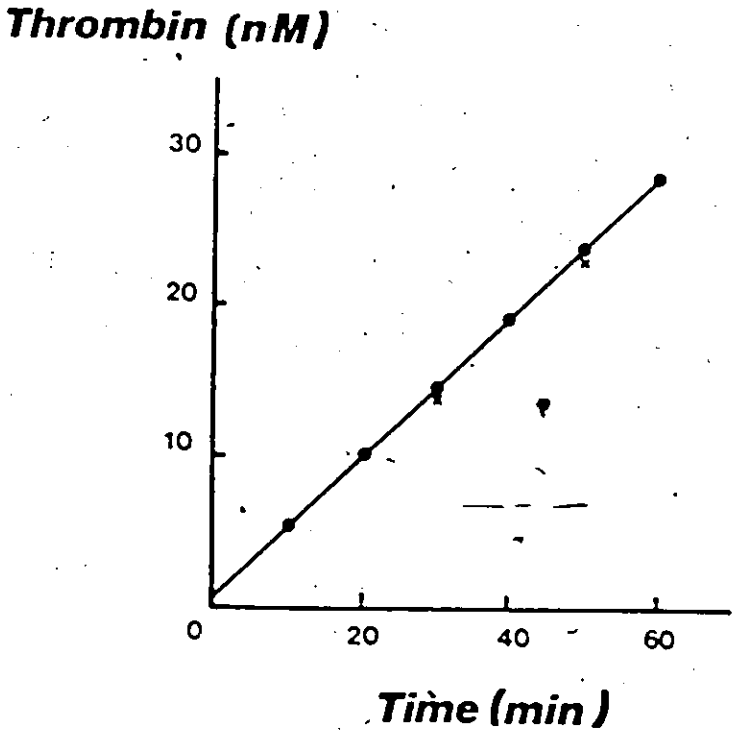

Thrombin (nM)
30
20
10
0
20
40
60
Time (min)

Fig. 26 Effect of F1.2 on thrombin generation in a purified protein system composed of 1 nM factor Va, 1 nM factor Xa, 10 mM $CaCl_2$ and 1 μM prothrombin. With no F1.2 (x—x); with 4 μM F1.2 (o—o). For details, see Methods section 2.14.6. Each data point is the mean of 2 determinations.

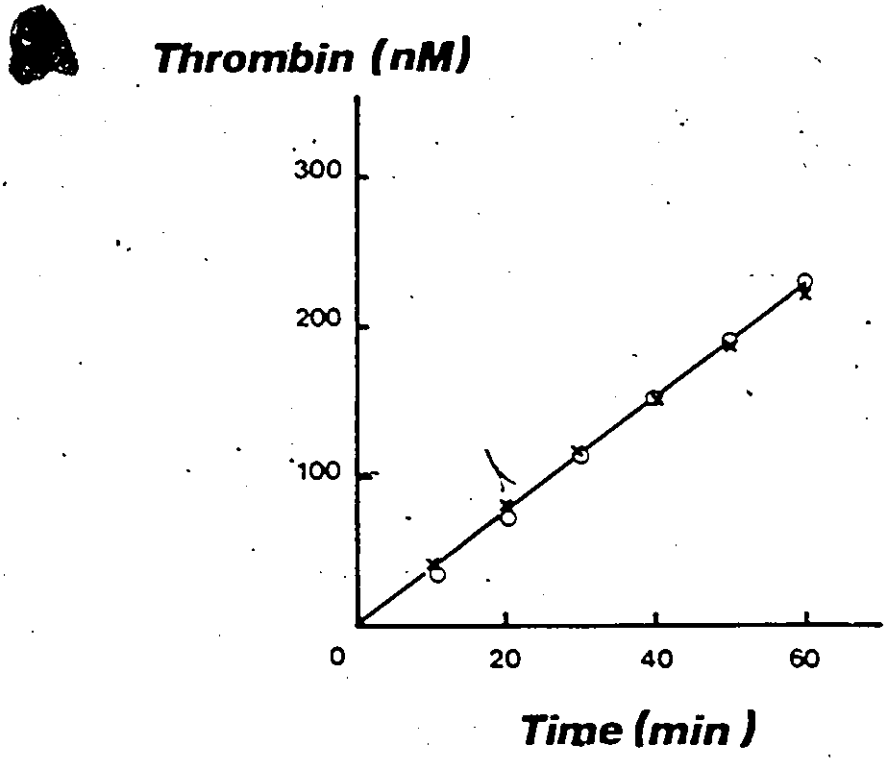

Thrombin (nM)
300
200
100
0
20
40
60
Time (min)

Fig. 27 Effect of F1.2 on thrombin generation in a purified protein system composed of phospholipid (60 ng organic phosphate/mL), 10 nM factor Xa, 10 mM $CaCl_2$ and 1 μM prothrombin. With no F1.2 (x—x); with 2 μM F1.2 (□—□); with 4 μM F1.2 (Δ—Δ); with 8 μM F1.2 (o—o). For details, see Methods section 2.14.6. Each data point is the mean of 2 determinations.

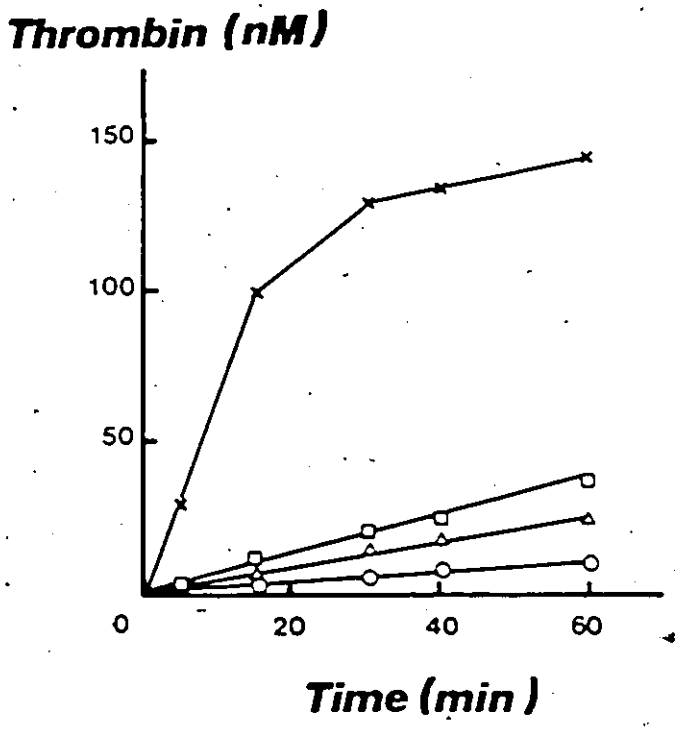
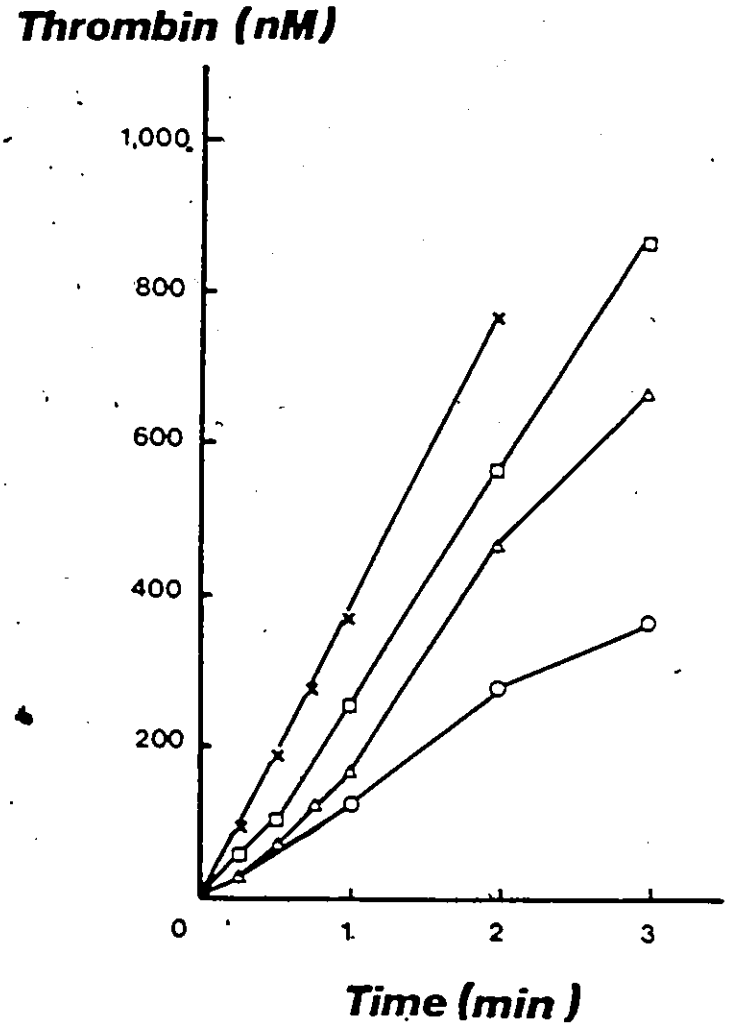

Thrombin (nM)
150
100
50
0
20
40
60
Time (min)

Fig. 28 Effect of F1.2 on thrombin generation in a purified protein system composed of phospholipid (60 μg organic phosphate/mL), 1 nM factor Va, 1 nM factor Xa, 10 mM $CaCl_2$ and 1 μM prothrombin. With no F1.2 (x—x); with 2 μM F1.2 (□—□); with 4 μM F1.2 (Δ—Δ); with 8 μM F1.2 (o—o). For details, see Methods section 2.14.6. Each data point is the mean of 2 determinations.


Thrombin (nM)
1,000
800
600
400
200
0
1
2
3
Time (min)

of factor Va decreases this anticoagulant activity of F1.2, thus, suggesting that factor Va interacts more efficiently with prothrombin than F1.2.

3.4. ANTITHROMBOTIC PROPERTIES OF FRAGMENT 1.2 IN VIVO

Thus far, it has been demonstrated that F1.2 could inhibit *in vitro* coagulation. However, whether F1.2 exerted any anticoagulant effect *in vivo* remained to be answered. Previously, Schneider (1947) and Thomas (1947) demonstrated independently that mice were killed by intravenous injections of tissue factor. Further, this tissue factor-induced death could be prevented by treatment with heparin or incubation of tissue factor with serum. Therefore, to test if F1.2 could act as an antithrombotic agent, *in vivo*, the protective effect of F1.2 in mice injected with lethal doses of tissue factor was determined. The quantitative mouse assay system was performed as described in Methods section 2.15. Thromborel S, a human placental tissue factor, which gave 14.6 ± 0.5 s prothrombin time, was assigned 1.0 unit/mL tissue factor activity. Different amounts of placental tissue factor were injected intravenously into mice of 20 to 25 grams body weight, and the minimum lethal dose was found to be 0.2 units of tissue factor (Table VII). The protective effect of heparin against lethal doses of placental tissue factor in this mouse assay system was confirmed as it was observed that 6 units (40 µg) of heparin

Table VII Effect of intravenous administration of different amounts of tissue factor (TF) into mice.

TF (units)	No. Live	No. Dead	Total No.	% Survival
0.02	6	0	6	100
0.05	3	3	6	50
0.1	4	2	6	67
0.2	0	6	6	0

protected all the mice from the lethal effect of injected tissue factor injections (Table VIII). Preliminary experiment indicated that injections of prothrombin F1.2 at 5 to 500 μg per mouse had no apparent adverse effect on the mice. The antithrombotic effect of F1.2 was assessed by administration of F1.2 with the tissue factor extract (Table IX). The degree of protection was a function of the dosage of F1.2; and 100% protection against a lethal dose of tissue factor was achieved with 500 μg F1.2.

In another experiment to assess the antithrombotic effect of F1.2, it was observed that when 500 μg of F1.2 was injected 30 min before the administration of lethal tissue factor, 67% of the mice survived.

The protective effect of F1.2 in a further survival experiment was performed to study the antithrombotic properties of F1.2. Using human thrombin as the thrombotic agent in mice, an intravenous dose of 20 μg (40 units) of thrombin killed all the mice (Table X). The simultaneous injection of 500 μg F1.2 with thrombin prevented 89% of the mice from thrombin-induced death (Table XI). Similar to the tissue factor experiment, 6 units of heparin was effective in protecting all the mice from thrombin-induced death (Table XII).

When the effects of factor Xa and cephalin in the mouse

Table VIII Effect of intravenous administration of mixtures of different amounts of heparin and lethal doses of tissue factor (0.2 units) into mice.

Heparin(units)	No. Live	No. Dead	Total No.	% Survival
0	0	6	6	0
3.0	2	4	6	30
6.0	6	0	6	100

Table IX Effect of intravenous administration of mixtures of different amounts of prothrombin F1.2 and lethal doses of tissue factor (0.2 units) into mice.

F1.2 (μg)	No. Live	No. Dead	Total No.	% Survival
0	0	6	6	0
50	0	6	6	0
100	1	5	6	17
200	3	3	6	50
300	3	3	6	50
400	3	3	6	50
500	6	0	6	100

Table X Effect of intravenous administration of different amounts of human thrombin into mice.

Thrombin (µg)	No. Live	No. Dead	Total No.	% Survival
5	3	0	3	100
10	3	0	3	100
15	3	0	3	100
20	0	3	3	0

Table XI Effect of intravenous administration of mixtures of different amounts of prothrombin F1.2 and lethal doses of human thrombin (20 μg) into mice.

F1.2 (μg)	No. Live	No. Dead	Total No.	% Survival
0	1	8	9	11
500	8	1	9	89

Table XII Effect of intravenous administration of mixtures of different amounts of heparin and lethal doses of human thrombin (20 μg) into mice.

Heparin(units)	No. Live	No. Dead	Total No.	% Survival
0	0	6	6	0
0.375	1	5	6	17
0.75	1	5	6	17
1.5	4	2	6	67
3.0	5	1	6	83
6.0	6	0	6	100

assay system were investigated, it was observed that the mice could tolerate intravenous injections of 1 to 40 μg of factor Xa or 0.06 to 1.2 μg of cephalin (6 μg of organic phosphate/mL) with no adverse effects. However, when factor Xa and cephalin were used as a mixture, all the mice died upon intravenous injections of 1 μg of factor Xa and 0.18 μg of cephalin (Table XIII). The effects of heparin and F1.2 against lethal doses of factor Xa and cephalin administration in mice were then studied. It was observed that 0.75 units of heparin protected all the mice against lethal doses of factor Xa and cephalin, but 500 μg of F1.2 did not protect any of the mice against lethal doses of factor Xa and cephalin (Table XIV and XV).

Table XIII Effect of intravenous administration of mixtures of different amounts of human factor Xa and cephalin (PL) into mice.

Xa (μg)	PL (μg)	No. Live	No. Dead	Total No.	% Survival
40.0	0	6	0	6	100
0	1.20	6	0	6	100
0.1	0.18	6	0	6	100
0.5	0.60	6	0	6	100
1.0	0.06	2	4	6	33
1.0	0.12	2	4	6	33
1.0	0.18	0	6	6	0
1.0	0.24	0	6	6	0
1.0	0.30	0	6	6	0

Table XIV Effect of intravenous administration of mixtures of different amounts of heparin and lethal doses of human Xa (1 μg) and cephalin (0.18 μg) into mice.

Heparin(units)	No. Live	No. Dead	Total No.	% Survival
0	0	6	6	0
0.15	0	6	6	0
0.375	4	2	6	67
0.75	6	0	6	100
6.0	6	0	6	100

Table XV Effect of intravenous administration of mixtures of different amounts of prothrombin Fl.2 and lethal doses of human Xa (1 µg) and cephalin (0.18 µg) into mice.

Fl.2 (µg)	No. Live	No. Dead	Total No.	% Survival
0	0	6	6	0
100	0	6	6	0
300	0	6	6	0
500	0	6	6	0

CHAPTER 4

DISCUSSION

Although purification of prothrombin F1.2 was reported several years ago, isolation of the fragment has been tedious. One approach adopted by several groups of researchers was from the initial purification of prothrombin (Esmon et al., 1974; Govers-Riemslag et al., 1985). Purified prothrombin was then activated with factor Xa and $CaCl_2$, with or without factor V and phospholipids, to form F1.2. As a result of the activation process, other products of prothrombin activation, such as prethrombin 2, and F1 and F2, were also produced. Consequently, purification of F1.2 requires additional chromatographic steps to separate F1.2 from factor Xa and other prothrombin activation products in the reaction mixture. Another approach was that reported by Aronson et al. (1977) which utilizes the adsorptive properties of F1.2 to DEAE-cellulose and hydroxylapatite; but the preparation obtained was not homogenous (Lau et al, 1979). The procedure for the purification of F1.2 reported in this study is relatively simple, and F1.2 was prepared without prior purification for prothrombin. Preparations were effectively

homogeneous as judged by electrophoretic, ultracentrifugation and immunological studies. The overall yield of F1.2 was about 7% if we assume all the prothrombin in the starting plasma was converted to F1.2. Final preparations had no detectable antiproteinase activities and were free of thrombin or factor Xa activities as measured by amidolytic assay systems. In addition, the preparations could be administered intravenously into mice with no apparent adverse effects.

4.1. IN VITRO ANTICOAGULANT PROPERTIES OF FRAGMENT 1.2

The in vitro experiments demonstrate that human prothrombin F1.2 possesses anticoagulant properties. The mode of action of F1.2 was studied in different reaction systems. Clotting of human plasma induced by tissue factor or activated partial thromboplastin reagent was inhibited by exogenous human F1.2 in a dose response manner. Using a defibrinated plasma system, a delay in thrombin generation was observed. The mechanism of inhibition on coagulation by F1.2 was not related to direct inhibition of tissue factor activity nor direct inhibition of factor X activation; instead F1.2 exerts its anticoagulant effect by interfering with phospholipid interactions in prothrombin activation by prothrombinase

complex. Thus, the inhibition is probably due to the gamma-carboxyglutamic acid containing region of prothrombin F1.2 competing with prothrombin and/or factor Xa for phospholipid binding sites (Gitel et al., 1973; Dombrose et al., 1979). Bovine prothrombin F1.2 has also been reported to inhibit prothrombin activation at the level of phospholipid in a kinetic study with purified components (Govers-Riemslag et al., 1985).

While F1.2 possesses the F2 domain which interacts with factor Va, prothrombin activation by factors Xa and Va in the absence of phospholipid was not inhibited by F1.2. In addition, factor Va could modulate the inhibitory effect of F1.2 on prothrombin activation by factor Xa and phospholipid. These observations are consistent with data published for the bovine system (Govers-Riemslag et al., 1985); and can be explained simply by assuming that factor Va can react better with prothrombin in prothrombinase complex assembly than with F1.2, per se. Studies to compare the interactions of factor Va with prothrombin and F1.2 in relation to factor Xa and phospholipid will undoubtedly provide direct information on this subject.

4.2. IN VIVO ANTITHROMBOTIC PROPERTIES OF FRAGMENT 1.2

The Wessler rabbit stasis model has often been used for assessing the thrombogenic and antithrombogenic potential of various materials (Wessler et al., 1959; Wessler, 1962). While the assay takes advantage of the ability of stasis to markedly potentiate intravascular coagulation and provide information about thrombosis at a localized site in vascular stasis, it does not deal with coagulation in a free flowing vascular system. Therefore, the mouse model was chosen for in vivo studies as it provides a physiologic environment for studying thrombosis and hemostasis. In addition, experiments with mice can be performed more economically than experiments with rabbits.

The clinical use of heparin in the prevention and treatment of thrombosis has a long history and its effectiveness is well documented. In this study, the antithrombotic effects of prothrombin F1.2 was investigated in parallel with heparin and heparin was used as a reference to compare antithrombotic efficacy of F1.2 in a mouse model.

The results of the in vivo antithrombotic experiments indicate that prothrombin F1.2 could modulate the effects of tissue factor initiated intravascular coagulation in a mouse

model. While 500 μg of exogenous human F1.2 was required to provide 100% protection against a lethal dose of tissue factor, administration of 100 μg of F1.2 protected 17% of the mice from tissue factor-induced death. Since the total plasma volume of a 20 to 25 g mouse is estimated at 0.8 to 1.0 mL (Wang, 1959), theoretically, 100 μg of F1.2 corresponds to the maximum amount of F1.2 that could be generated if all the prothrombin in 1.0 mL of plasma were converted to F1.2. Thus, administration of human prothrombin F1.2 does protect the mice although relatively high concentrations are required (500 μg/mL or 14 μM for 100% protection). In comparison, a relatively high dose of heparin (6 units or 40 μg or 3 μM) was also required to protect all the mice from tissue factor-induced death. It has been demonstrated that prevention of extension of thrombosis could be achieved with a heparin level of 0.3 to 0.6 units/mL in a rabbit stasis model (Chiu et al., 1977). In addition, infusions of heparin (5 to 20 units/Kg) in a rabbit stasis model (equivalent to 0.1 to 0.4 units/20 g mouse) significantly reduced the extent of thrombosis induced by injections of activated coagulation factors and tissue factor (Gitel et al., 1977; Gitel and Wessler, 1979).

Several factors may account for the relatively large amount of human prothrombin F1.2 required to protect the mice

in this model. First, the lethal amount of tissue factor administered constitutes an unusually severe challenge which cannot be totally neutralized with physiologic concentrations of naturally occurring anticoagulants. Second, prothrombin F1.2 formed on the surface of the injured endothelium may be more effective in inhibiting coagulation than the infused preparations. Third, possible generation of other thrombosis promoting components, e.g., factors Xa and Va, caused by initiation of coagulation may decrease the effectiveness of F1.2. Fourth, infused human prothrombin F1.2 may be less effective in inhibiting thrombosis in a different species as ability of human F1.2 to compete with mouse prothrombin is not known.

It is of interest to note that human prothrombin F1.2 could also play a role in modulating human thrombin-induced death in the mouse model, as prothrombin F1.2 does not inhibit proteolytic activities of thrombin in vitro. The dosages of human prothrombin F1.2 required to prevent thrombin-induced death were similar to that required for prevention of tissue factor-induced death. It can be hypothesized that in order for thrombin to cause death of mice, thrombin has to prime the endothelium and platelets, and overcome inherent natural anticoagulant mechanisms, e.g., antiproteinases such as

antithrombin III, α_1-proteinase inhibitor, and α_2-macroglobulin. Thus, it is possible that the thrombin administered intravenously exert its lethal action on mice through further generation of thrombin, in vivo, from positive feedback reactions via activation of factors V and VIII (Mann and Lundblad, 1987). F1.2 was effective in inhibiting this further generation of thrombin, in vivo, by interfering with prothrombin activation, and was therefore able to prevent death of mice.

In this study, it was observed that infusion of factor Xa alone, even at concentrations more than 4-fold that of normal factor X concentration did not cause intravascular coagulation in mice. Normal factor X concentration in human plasma is 7 to 8 µg/mL (Miletich et al., 1981). A source of phospholipid, such as cephalin, was required for physiologic concentration (1 µg) of factor Xa to exert lethal effect in mice. This synergistic lethal effect of factor Xa and cephalin may be attributed to the enhancement effect of phospholipid on factor Xa-catalyzed activation of prothrombin and factor V (Foster et al., 1983). Barton et al. (1970) also observed that factor Xa complexed with phospholipid manifested increased thrombotic activity in a rabbit stasis model. The amount of heparin (0.75 units of heparin) required to inhibit this

cephalin and factor Xa induced lethality in mice was substantially less than that (6 units of heparin) required to inhibit either tissue factor or thrombin induced death. This result may reflect the difference in heparin requirement for inhibiting the synergistic effect of 1 μg factor Xa and cephalin versus inhibiting activity of tissue factor or 20 μg thrombin. Yin (1974) has shown that adsorption of factor Xa to phospholipid surfaces protects the enzyme from inhibition by antithrombin III, and heparin could prevent the phospholipid protection. These results also illustrate the versatile mode of action of heparin as an antithrombotic agent in preventing intravascular coagulation.

The observation that 500 μg of F1.2 did not inhibit cephalin and factor Xa induced death in mice suggest that when there was adequate factor Xa formed as a complex with cephalin, the factor Xa-cephalin complex was capable of causing intravascular coagulation which could not be inhibited effectively by prothrombin F1.2. Together with other *in vivo* experiments, this observation suggest that the amounts of factor Xa and procoagulant phospholipids generated *in vivo* from intravenous injections of lethal doses of tissue factor or thrombin were limited. Further, it is possible that increases in procoagulant phospholipid concentrations could modulate the

antithrombotic effect of F1.2, which is consonant with the observation in vitro that it competes for phospholipid binding sites. In addition, it has been reported that factor Xa and phospholipids protect factor Va from inactivation by activated protein C (Nesheim et al., 1982). Hence, it is possible that the unique synergistic lethal action of factor Xa and cephalin was not effectively inhibited by F1.2 as the results of experiments, in vitro, showed that factor Va could reduce the effectiveness of F1.2 in inhibiting prothrombin activation.

The results of the in vitro and in vivo experiments, presented in this thesis, provide strong evidence in support of the potential anticoagulation and antithrombotic properties of prothrombin F1.2 in modulating both extrinsic and intrinsic pathways of coagulation. Since F1.2 is a physiologic metabolite and has antithrombotic effects it may be of interest to contemplate its use in prophylactic treatment of patients at risk of thromboembolic events.

SUMMARY

The aim of these studies was to develop a simple and efficient method for purification of human prothrombin F1.2 from plasma and to determine the anticoagulant and antithrombotic properties of prothrombin F1.2 towards a better understanding of some of the mechanisms that could be responsible for the modulation of coagulation.

The aspects of this thesis which present new developments and have advanced our knowledge in relation to thrombosis and hemostasis are :

1. A new method for preparing prothrombin F1.2 from human plasma. The features of this method which are distinct from those used by other investigators or improvements over earlier methods are : (i) preparation of purified prothrombin F1.2 from plasma without resorting to prior purification for prothrombin, (ii) use of benzamidine to inhibit degradation of F1.2, (iii) use of benzamidine-Sepharose to remove activated coagulation factors, and (iv) use of Cibacron blue-agarose instead of gel filtration to remove high molecular weight contaminants.

2. Human prothrombin F1.2 has anticoagulant activities

and its mechanism of action is inhibition of prothrombin activation by prothrombinase complex at the level of phospholipid interactions. The present observation is believed to constitute the first report of anticoagulant properties of human prothrombin F1.2.

3. Human prothrombin F1.2, in the presence or absence of factor VIIa and factor Xa, does not inhibit tissue factor activity in vitro.

4. Human prothrombin F1.2 does not inhibit factor X activation in vitro.

5. Human prothrombin F1.2 has antithrombotic activities in preventing lethal effect of tissue factor in mice, and preventing lethal effect of thrombin in mice.

6. Human prothrombin F1.2 is not effective in preventing factor Xa and cephalin induced lethality in mice.

Collectively, these findings demonstrate that human prothrombin F1.2 has anticoagulant and antithrombotic properties, and probably through its gamma-carboxyglutamic acid region confers upon the activation fragment of prothrombin the capacity to modulate coagulation in vitro and in vivo.

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